Biochemical Pharmacology, Vol. 51, pp. 931–940, 1996.

ELSEVIER

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00050-0

Growth Arrest and Non-apoptotic Cell Death Associated with the Suppression of c-*myc* Expression in MCF-7 Breast Tumor Cells Following Acute Exposure to Doxorubicin

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ABSTRACT. In the MCF-7 human beast adenocarcinoma cell line, acute exposure to 1 μM doxorubicin inhibited cell proliferation by ~75%. Analysis of cell cycle distribution indicated that within 24 hr, the G_2/M fraction increased more than 3-fold and the S-phase population declined by >50%. In addition to growth arrest, there was an ~40% reduction in the viable cell population after 72 hr. Gel electrophoretic resolution of low molecular weight DNA immediately after exposure of cells to doxorubicin failed to demonstrate "laddered" oligonucleosomal profiles associated with apoptosis. The absence of intracellular DNA fragments or release of fragmented DNA into the incubation medium was confirmed by spectrofluorophotometry over a 72 hr interval following exposure of cells to 1 μM doxorubicin. In addition, there was no evidence of the morphological features associated with apoptosis during this period. Acute exposure to 1 μM doxorubicin also produced a transient increase in c-*myc* message expression (within the first hour) followed by a decline to 70% of control levels within 2–4 hr. The reduction in c-*myc* mRNA levels was concentration dependent and corresponded closely with growth arrest (as well as with inhibition of DNA synthesis). These findings (as well as similar reports demonstrating a correspondence between reduced c-*myc* expression and growth inhibition by VM-26 and m-AMSA in MCF-7 cells) suggest that the down-regulation of c-*myc* expression may reflect perturbations in regulatory processes contributing to growth arrest in MCF-7 cells exposed to topoisomerase II inhibitors. BIOCHEM PHARMACOL 51;7:931–940, 1996.

KEY WORDS. c-*myc*; doxorubicin; MCF-7; breast cancer

Although doxorubicin is widely recognized for its clinical utility in the treatment of breast cancer [1], the basis for its antiproliferative and cytotoxic effects has not been defined conclusively. In a previous report from this laboratory, it was suggested that a hierarchy of cellular perturbations may be responsible for growth arrest in MCF-7 breast tumor cells acutely exposed to doxorubicin [2]. At relatively low concentrations (0.05 through 0.5 μM), doxorubicin interferes with DNA unwinding [2]. At a concentration of 1 μM, doxorubicin inhibits topoisomerase II, as indicated by the induction of protein-associated strand breaks in DNA [2]. Finally, supra clinical concentrations (5 μM) of doxorubicin produce non-protein associated DNA-strand breaks, suggestive of free-radical generation [2]. In contrast, chronic exposure to a concentration (50 nM) that is typically sustained in the peripheral blood for up to 12 hr following i.v. administration [3, 4] appears to engage a unique growth arrest/cell death pathway involving damage to nascent DNA, endoreduplication of DNA, and differentiation-induction associated with a gradual reduction in expression of the c-*myc* oncogene [5].

Our principal interest in the c-*myc* oncogene is based upon its critical role in growth regulation in the MCF-7 breast tumor cell line [6, 7], and its overall contribution to the proliferative activity of breast tumor cells [8]. c-*myc* has been shown to be deregulated or overexpressed in many clinical breast tumor samples [9], while amplification of c-*myc* is associated with early relapse and poor response [10]. The Myc protein is also thought to represent a component of the apoptotic cell death pathway [11, 12].

We have reported previously that the topoisomerase II inhibitors VM-26§ and m-AMSA produce a concentra-

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Received 12 May 1995; accepted 20 November 1995.

§ *Abbreviations:* VM-26 (teniposide), 4′-demethylepipodophyllotoxin-4-(4,6-*O*-thenylidene-β-D-glucopyranoside); m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; SSC, standard saline citrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

tion-dependent reduction in the expression of c-*myc*, which precedes and correlates with growth arrest in MCF-7 breast tumor cells [13, 14]. Recent studies in our laboratory have demonstrated that a similar relationship between the suppression of c-*myc* expression and growth arrest is evident for ionizing radiation in the MCF-7 breast tumor cell line.[‖] These observations suggest that c-*myc* could play the role of a DNA-damage response gene in this breast tumor cell line.

The present studies were designed to assess the influence of acute exposure to 1 μM doxorubicin on the expression of the c-*myc* gene as well as the induction of growth arrest and apoptotic cell death in the MCF-7 breast tumor cell line. Our studies indicate a time- and concentration-dependent reduction in c-*myc* expression which correlates closely with growth arrest. Although we also observed a significant reduction in the number of viable cells, we found no morphological or biochemical evidence of apoptotic cell death or of DNA fragmentation. These findings indicate that acute exposure to doxorubicin results in both cytostasis and (delayed) death of MCF-7 breast tumor cells by a non-apoptotic pathway. Furthermore, it appears that growth arrest induced by topoisomerase II inhibitors in this cell line may be related to suppression of c-*myc* expression. It will be necessary to extend these observations to other breast tumor cell lines in order to establish a relationship between c-*myc* expression and the cellular response to DNA damage.

MATERIALS AND METHODS

Materials

DMEM (56-439) was obtained from Hazelton Research Products, Denver, PA; L-glutamine, penicillin (10,000 U/mL), streptomycin (10 mg/mL) and fetal bovine serum were obtained from Whittaker Bioproducts, Walkersville, MD; defined bovine calf serum was obtained from Hyclone Laboratories, Logan, UT. Trypsin-EDTA and agarose were obtained from GIBCO BRL (Gaithersburg, MD). Doxorubicin, DMSO, thymidine, trichloroacetic acid, *bis*-benzimide trihydrochloride (Hoechst 32258), proteinase K, and MTT were obtained from the Sigma Chemical Co., St. Louis, MO. The radiolabeled compounds [^{3}H]thymidine and [α-^{32}P]dCTP were obtained from ICN Radiochemicals, Irvine, CA, and DuPont NEN Research Products, Boston, MA, ATP, CTP, and GTP were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. All other chemicals were reagent or molecular grade, as appropriate.

The c-*myc* probe, an *Eco* RI/*Cla* I fragment of pmC41 3RC containing the third exon of the human c-*myc* gene, was provided by Dr. Eric Westin of the Medical College of Virginia. Genomic or complementary DNA probes for GADPH were obtained from the American Type Culture Collection, Rockville, MD. The nick-translation kit was obtained from GIBCO BRL.

Cell Line

The MCF-7 breast tumor cell line was provided by the laboratory of Dr. Kenneth Cowan at the National Cancer Institute, Bethesda, MD. Cells were maintained in DMEM (Hazelton Research Products) supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY), 5% defined bovine serum (Hyclone Laboratories), glutamine (29.2 mg/100 mL), and penicillin/streptomycin (0.5 mL/100 mL) (Whittaker Bioproducts.

Growth Inhibition

The capacity of doxorubicin to interfere with the growth of the MCF-7 breast tumor cell line was determined using the MTT assay [15], as described in detail previously [16]. Briefly, cells subcultured at a density of 1×10^{-4} cells/mL in 96-well microplates (Costar, Cambridge, MA) were incubated with doxorubicin for either 2 or 4 hr at 37°, washed free of drug, and incubated in fresh medium for an additional 72 hr. Medium was removed by gentle inversion of the microplate, and the cells were washed with PBS, prior to incubation with 100 μL of MTT solution (2 mg/mL MTT in PBS; filtered as needed). MTT is converted to a blue formazan product by mitochondrial succinate dehydrogenase. This product was eluted from cells by addition of 100 μL of DMSO, and absorbance, at 540 nm, was determined using an EL310 EIA autoreader (Biotek Instruments, Burlington, VT). It should be noted that this assay fails to distinguish between growth arrest and a reduction in cell number due to cell death.

Determination of Viable Cell Number

To determine the capacity of doxorubicin to produce cell death, the reduction in viable cell number was assessed after 72 hr. MCF-7 cells subcultured at a density of 1.5×10^{-4} cells/mL in 75 cm^2 T flasks (Costar) were incubated with 1 μM doxorubicin for 4 hr at 37°, washed free of drug, and incubated in fresh medium for an additional 72 hr. At appropriate intervals the drug was aspirated, and the cells were washed with ice cold PBS (pH 7.4). Cells were released from flasks by incubation with trypsin (0.05 mg/mL)/EDTA (0.02 mg/mL) for 5 min at 37°, collected in ice-cold PBS (pH 7.4), and centrifuged at 4°. Cell pellets were resuspended in 300–500 μL of ice-cold PBS, and aliquots were mixed with trypan blue. Cells were loaded on a hemocytometer, and viable cell number was determined based on exclusion of trypan blue dye.

Inhibition of DNA Synthesis

The influence of doxorubicin on DNA synthesis in MCF-7 breast tumor cells was determined based on the incorpora-

[‖] Watson NC, Orr MS, Fornari FA and Gewirtz DA, Manuscript in preparation.

tion of [^{3}H]thymidine into acid-precipitable material, as described in detail previously [16]. Briefly, after exposure to drug in 24-well plates, drug was aspirated, and cells were washed in DMEM and incubated with [^{3}H]thymidine. At 10-min intervals (over a total time frame of 40 min), the radiolabel was aspirated, and the cells were washed with DMEM and detached from the plastic substratum by incubation with trypsin (0.05 mg/mL)/EDTA (0.02 mg/mL) for 5 min at 37°. The suspended cells were transferred to ice-cold trichloroacetic acid, and the precipitated radiolabeled DNA was isolated on disc filters using a Millipore Filtration apparatus. Inhibition of DNA synthesis was determined by comparing the rates of [^{3}H]thymidine incorporation in control and drug-treated cells.

Light Microscopic Analysis of Cell Morphology

MCF-7 breast tumor cells in 75 cm^2 T flasks (Costar) were incubated with 1 μM doxorubicin for 4 hr at 37°, washed free of drug, and incubated in fresh medium for an additional 72 hr. At appropriate intervals, the drug was aspirated and the cells were washed with ice-cold PBS (pH 7.4) and released from flasks by incubation in trypsin (0.05 mg/mL)/EDTA (0.02 mg/mL) for 5 min at 37°. The cells were collected in ice-cold PBS (pH 7.4) and centrifuged at 4°. After resuspension, the cells were deposited on cytocentrifuge slides and stained with a 20% Wright–Giemsa stain.

Cell Cycle Analysis

MCF-7 breast tumor cells in 75 cm^2 tissue culture flasks were grown for 4 hr (37°, 5% CO_2, 100% humidity) in drug-free medium or medium containing 1 μM doxorubicin, washed free of drug, and incubated in fresh medium for an additional 72 hr. At the appropriate intervals, cells were washed once in 1× PBS and released from flasks by incubation with trypsin (0.05 mg/mL)/EDTA (0.02 mg/mL) for 5 min at 37°. Cells in trypsin were diluted with cold medium to prevent proteolysis, and the suspensions were pelleted at 400 *g* in a Beckman model TJ-6 refrigerated centrifuge (Beckman, Spinco Division, Palo Alto, CA). Cells were resuspended in 1.0 mL of fluorochrome staining solution containing propidium iodide (0.05 mg/mL), sodium citrate (3.8 mM), RNase B (7000 U/mL), and 0.1% Triton X-100 [17]. Stained cell nuclei were strained through 62 μm mesh filters into microfuge tubes and stored at 4° until the day of analysis. Before testing, cells were filtered using 35 μm mesh nylon. Nuclei were analyzed with an EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL) using the 488 nm line of an argon laser and standard optical emission filters. To exclude doublets, bitmap gating on histograms of integral versus peak fluorescence signals was employed. The resulting DNA distributions were analyzed for the proportion of cells in various stages of the cell cycle [18] using Cytologic Software (Coulter Electronics).

Spectrofluorophotometric Quantitation of DNA Fragments

Quantitative analysis of DNA fragmentation, permitting quantification of DNA fragments in both lysate and medium preparations, was performed using a miniaturized adaptation of *bis*-benzimide spectrofluorophotometry [19] as described previously [20]. As above, cells were incubated with 1 μM doxorubicin for 4 hr at 37°, washed free of drug and incubated in fresh medium. For measurement of cellular DNA fragments, pelleted cells were lysed by incubation in 0.1% Triton X-100 (fully hydrogenated)/5 mM Tris–HCl/20 mM EDTA, pH 8.0 (100 μL/10^6 cells) for 15 min, and the lysates were centrifuged at 30,000 *g* at 4° for 40 min. For measurement of DNA fragment release, aliquots of incubation medium were centrifuged at 20,000 *g*. The pellets were discarded, and the presence of non-sedimenting DNA fragments in the supernatant was determined by dilution in modified TNE buffer (3 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 1.0 μg/mL *bis*-benzimide trihydrochloride (Hoechst 33258; Sigma), and monitoring net fluorescence in each sample (λ_{ex} = 365, λ_{em} = 460). Final DNA values were calculated in terms of highly purified calf thymus DNA calibration standard; values for all such responses were measured as nanograms DNA recovered or released from 10^6 cells, and reflect the absolute amount of non-sedimenting, small molecular weight (i.e. ≤3000 bp) fragments of DNA present in lysate and medium preparations.

Agarose Gel Electrophoresis

Qualitative analysis of DNA fragments was performed by conventional agarose gel electrophoresis as described previously [20]. Cells were incubated with 1 μM doxorubicin for 4 hr at 37°, washed free of drug, and incubated in fresh medium. Cells were released from flasks by incubation with trypsin (0.05 mg/mL)/EDTA (0.02 mg/mL) for 5 min at 37°, collected in ice-cold PBS (pH 7.4), and centrifuged at 4°. Pelleted cells were lysed by incubation for 15 min in 0.1% Nonidet P-40, 10 mM Tris–HCl, 25 mM EDTA, pH 7.4, and dispersed with gentle mechanical agitation; the lysate was then treated with 500 μg/mL proteinase-K at 55° for 16 hr. The deproteinated extract was centrifuged at 30,000 *g* for 75 min at 4°, and the pellet was discarded; the supernatant was treated with ribonuclease-A (0.1 mg/mL; Sigma) at 37° for 18 hr. Small molecular weight DNA residing in the final extract was resolved by electrophoresis at 85–115 V for 90–240 min on 2.25% agarose gels impregnated with ethidium bromide. DNA fragments were visualized under UV light. Multiple DNA molecular weight reference preparations (100-, 123-, and 1000-bp ladders; GIBCO BRL) routinely were run in parallel to facilitate assessment of the relative size of oligonucleosomal DNA fragments.

Gene Expression by Northern Blotting

Expression of mRNA for c-*myc* and for GAPDH was determined by standard northern blotting. RNA was isolated

from cells as previously described [21]. Briefly, after incubation, drug was removed, and cells were washed with PBS (pH 7.4) and lysed in 4 M guanidine isothiocyanate and 20% sodium lauryl sarcosine. RNA was separated by ultracentrifugation through a 5.7 M cesium chloride cushion at 41,000 *g* for 20 hr at 20°. RNA pellets were washed in 95% ethanol and resuspended in Milli Q water. Ten micrograms of RNA was denatured in 0.02 M morpholino propane sulfonic acid, pH 7.0, 5 mM NaAc, 1 mM EDTA, 2.2 M formaldehyde, and 50% formamide and separated on a 6.6% formaldehyde, 1% agarose gel [22]. Blotting was carried out using Nytran transfer membranes (Schleicher & Schuell, Keene, NH).

Probes were radiolabeled using a nick translation kit (GIBCO BRL) and hybridized to membranes for 16–20 hr at 42° in the presence of 50 mM sodium phosphate, pH 6.5, 5× Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinyl pyrrolidine) 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 0.1% SDS, yeast RNA (250 μg/mL), 50% formamide, and 10% dextran sulfate [23]. Filters were washed accordingly at 42° in 2× SSC and 0.2% SDS followed by 2× SSC and 0.2% SDS for approximately 40 min before autoradiography and Betascope blot analysis (Betagen Corp., Waltham, MA).

Statistical Analysis

Statistical analysis was performed using Student's *t*-test. Drug effects that resulted in *P* values of 0.05 or below were considered statistically significant.

RESULTS

Effect of Doxorubicin on Cell Growth and Viability

Acute exposure of MCF-7 breast tumor cells to doxorubicin was demonstrated previously to produce a concentration-dependent inhibition of growth, with an IC_{50} of 0.1 μM [2]. At 1 μM doxorubicin, the concentration utilized throughout the present study, growth was reduced by 74.7 ± 2.5%, when compared to growth of untreated control cells. As indicated below, a separate analysis indicated that there was a reduction of approximately 40% in the viable cell population after 72 hr.

We have reported previously that acute exposure to 1 μM doxorubicin produces protein-associated single-strand breaks in bulk DNA [2]. Because exposure to agents that induce DNA damage generally results in a G_2/M-arrest [24–26], flow cytometric analysis was utilized to determine the influence of acute exposure to 1 μM doxorubicin, a level that is maintained in the plasma for several hours following i.v. bolus administration [3, 4] on cell-cycle progression. The results shown in Table 1, indicate that, within 24 hr, the G_2M fraction increased from 5.4 to 32.5%, the percentage of S-phase cells was reduced from 30.5 to 9.9%, and the G_0/G_1 fraction showed a slight decline. The untreated controls maintained a relatively uniform phase distribution throughout the experimental time frame (i.e. 23% S, 5% G_2M, and 72% G_1).

TABLE 1. Effects of 1 μM doxorubicin on MCF-7 cell cycle traverse

Cycle phase	Time (h)	Distribution of cells (%)		% of Control
		Control	Doxorubicin	
G_0/G_1	6	64.1	60.7	94.7
	24	71.6	57.6	80.5
	48	67.9	53.9	79.4
S	6	30.5	29.4	96.4
	24	22.9	9.9	43.3
	48	26.8	17.2	64.2
G_2/M	6	5.4	9.9	183.3
	24	5.5	32.5	590.1
	48	5.3	28.9	545.3

Adherent cultures of MCF-7 cells were incubated for 4 hr in the absence or presence of doxorubicin (1 μM), and the distribution of cells in various phases of the cell cycle (i.e., G_0/G_1, S, G_2/M) was then determined by flow cytometry as described. Values are expressed as a percentage of untreated controls.

Effect of Doxorubicin on Steady-State c-myc mRNA levels

Previous work in our laboratory has demonstrated a close correspondence between inhibition of growth of MCF-7 cells and of c-*myc* expression after exposure to topoisomerase II inhibitors such as VM-26 and m-AMSA [13, 14]. We therefore assessed the influence of acute exposure to doxorubicin on c-*myc* expression. Figure 1 presents the results of northern blot analyses, demonstrating that exposure to 1 μM doxorubicin produced a transient increase followed by a time-dependent reduction of c-*myc* mRNA levels (with a maximal reduction observed at 3–4 hr), while expression of the "housekeeping gene," GADPH, remained essentially unaltered. (It should be emphasized that each time point after doxorubicin treatment is compared to its time-equivalent control in order to correct for random variations in c-*myc* expression.) Figure 2 demonstrates that acute exposure to doxorubicin produced a concentration-dependent reduction in c-*myc* mRNA levels. As reported previously in studies involving VM-26 and m-AMSA in MCF-7 breast tumor cells [13, 14], a close correlation was observed ($r^2 = 0.923$) between the extent of inhibition of c-*myc* expression (assessed after a 4-hr exposure to doxorubicin) and growth inhibition measured 72 hr after drug exposure (Fig. 3A). Figure 3B demonstrates that a similar correspondence was observed between the reduction in c-*myc* expression and the early inhibition of DNA synthesis ($r^2 = 0.962$). Data for concentration-dependent growth inhibition and for inhibition of DNA synthesis by doxorubicin in MCF-7 cells were generated previously [2].

It should also be noted that, in contrast to the enhancement of DNA synthesis previously reported in MCF-7 cells chronically exposed to a low (differentiation-inducing) concentration of doxorubicin [5], acute exposure to 1 μM

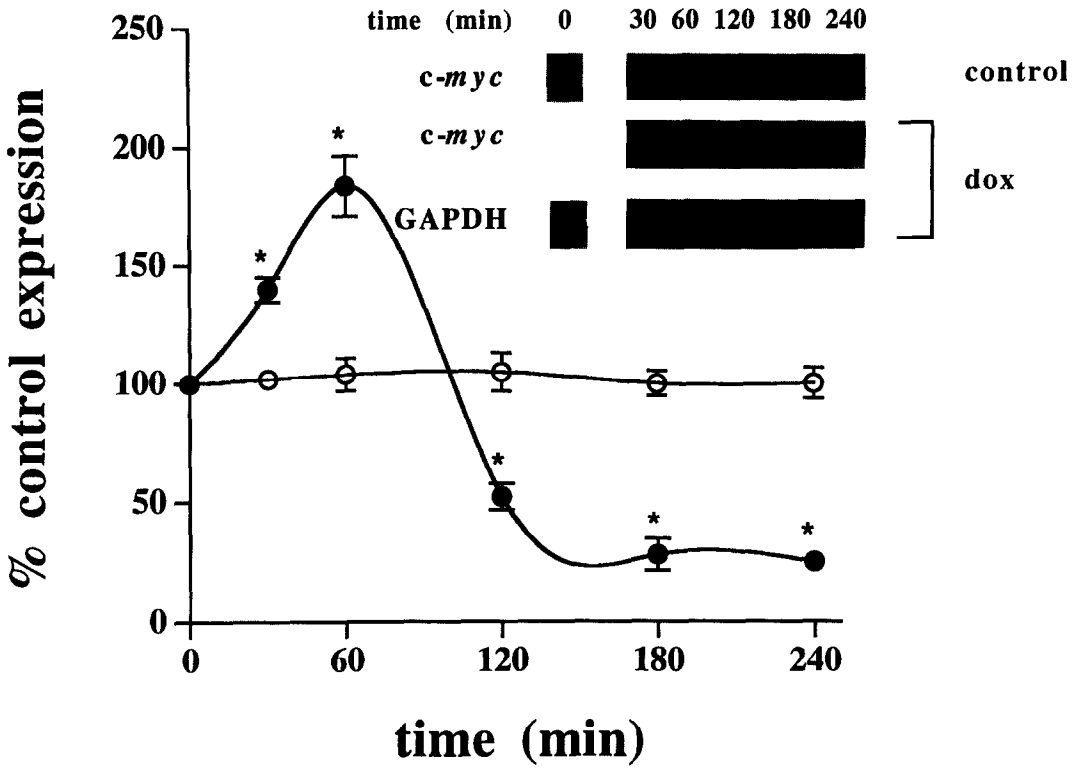

FIG. 1. Time-dependent effects of 1 μM doxorubicin on mRNA levels in MCF-7 breast tumor cells. A quantitative representation of the time-dependent effects of doxorubicin on c-*myc* (●) and GAPDH (○) mRNA levels is shown. The c-*myc* message expression is presented as a percent of time-equivalent controls. GAPDH expression is presented as a percent of the zero-time control. Data are the means ± SEM for 3 experiments. Key: (*) significant at $P < 0.05$. Inset: Autoradiographic display of the time-dependent changes in (a) c-*myc* expression in control cells; (b) c-*myc* expression in cells exposed to 1 μM doxorubicin; and (c) GAPDH expression in cells exposed to 1 μM doxorubicin.

doxorubicin resulted in a sustained inhibition of DNA synthesis over a period of 72 hr as measured by [^{3}H]thymidine uptake (not shown).

Influence of Doxorubicin on Cell Morphology and DNA Fragmentation

As doxorubicin has been reported to induce apoptosis [27, 28], we were interested in determining whether doxorubicin maintained the capacity to produce apoptotic cell death in a cell line where c-*myc* expression was suppressed. Consequently, the number of surviving cells was determined after acute exposure of a logarithmically growing population to 1 μM doxorubicin. Figure 4 indicates that there were minimal reductions in cell number after 24 hr, but that after 72 hr, cell number had declined by approximately 40%. Alterations in cellular morphology and DNA integrity of MCF-7 cells were examined following exposure to 1 μM doxorubicin. The typical appearance of MCF-7 cells at 24-hr intervals after a 2-hr exposure to 1 μM doxorubicin is shown in Fig. 5. While these cells clearly exhibited gross perturbations in morphology in response to doxorubicin, there was no indication of the morphological features commonly used to define apoptosis (e.g. chromatin condensation, disappearance of nucleoli, formation of membrane blebs, apoptotic bodies, and cell shrinkage) [29]. These cells also failed to show morphological evidence of necrotic cell death (e.g. loss of nuclear definition, distortion of organelle structure, cell swelling, and gross cytolysis) and instead, appeared to undergo autocatalysis, as indicated by the appearance of numerous perinuclear autocatalyic vacuoles, which has been observed in MCF-7 cells chronically exposed to doxorubicin [5].

To substantiate the non-apoptotic character of doxorubicin-induced cell death, MCF-7 cells were incubated in the absence or presence of doxorubicin (1 μM) for 2 hr, and damage to genomic DNA was evaluated by several criteria. Immediately after exposure to drug, agarose gel electrophoresis failed to demonstrate laddered electrophoretic profiles of oligonucleosomal DNA fragments indicative of apoptosis (not shown). Quantitative spectrofluorophotometry demonstrated neither intracellular accumulation nor release of low molecular weight double-stranded DNA fragments over an interval of 6–72 hr following drug exposure (Fig. 6). The absence of DNA fragmentation in the incubation medium is a particularly important observation in view of reports of apoptosis in adherent cells that have become detached from the culture plate [30, 31]. Further analysis of DNA frag-

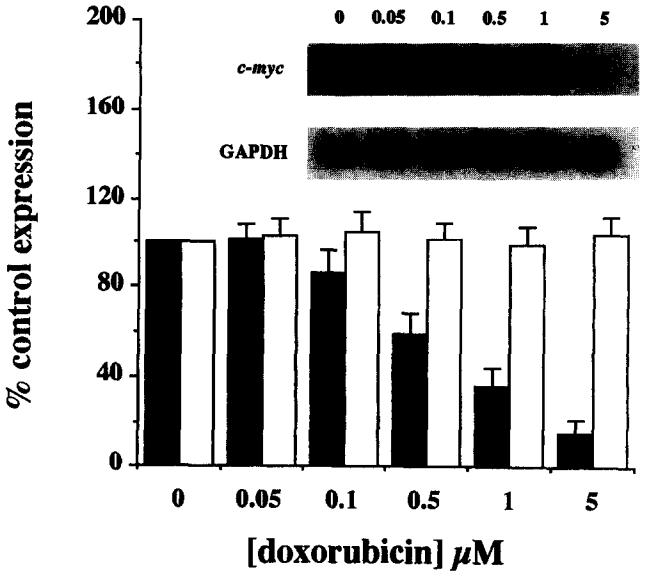

FIG. 2. Concentration-dependent effect of doxorubicin on mRNA levels in MCF-7 breast tumor cells. MCF-7 cells were exposed to various concentrations of doxorubicin for 4 hr and northern blot analysis was performed as described in Materials and Methods. A quantitative representation of the concentration-dependent effects of doxorubicin on c-myc (solid bars) and GAPDH (open bars) mRNA levels is shown. Data are the means ± SEM for 3 experiments. Inset: Autoradiographic display of the concentration-dependent changes in c-myc and GAPDH expression in cells acutely exposed to doxorubicin.

mentation by pulse-field gel electrophoresis failed to identify larger fragments generated in other tumor cell lines by agents such as TGF-β, TNF and ceramide [20, 32, 33] [i.e. 300-and 50-kbp fragments corresponding to, respectively, rosette and loop structures in static chromatin (not shown)].

DISCUSSION

Implications Relating to the Reduction in c-myc Expression by Doxorubicin

The studies presented in this report extend our previous findings indicating that topoisomerase II inhibitors are capable of down-regulating c-*myc* expression in MCF-7 breast tumor cells following acute drug exposure [13, 14]. In a previous work, we further demonstrated that suppression of c-*myc* expression by VM-26 occurs at the transcriptional level, and that the response is transient [34]. These findings raise the possibility that suppression of c-*myc* expression represents a component of a signal transduction pathway leading to growth arrest in breast tumor cells.

The early alterations in c-*myc* expression involved a transient increase within the first hour followed by a subsequent decline that was sustained and constant after 3 and 4 hr. The transient increase is difficult to explain, but may reflect the capacity of c-*myc* to respond to mitogenic signals [35]; that is, although doxorubicin inhibits tumor cell growth, Vichy and Tritton [36] have also reported a

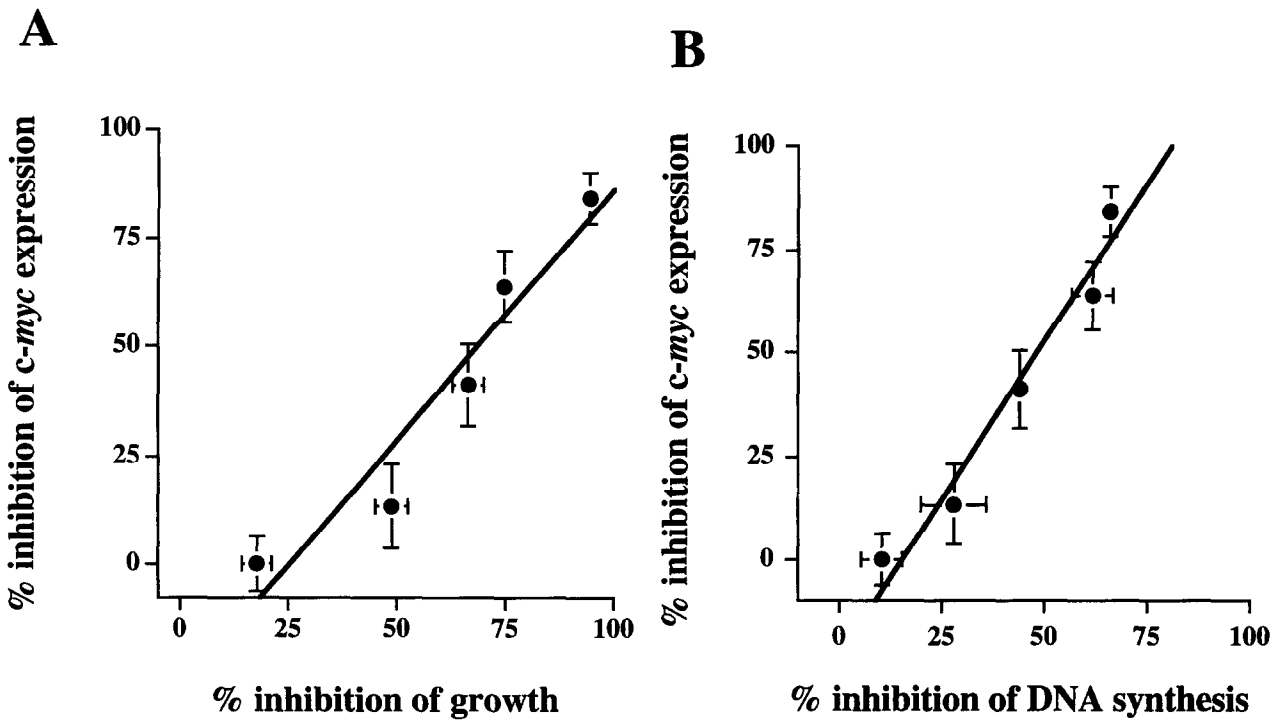

FIG. 3. Correlation between concentration-dependent reductions in c-myc mRNA levels and growth inhibition (A) and inhibition of DNA synthesis (B). In MCF-7 cells exposed to doxorubicin, growth inhibition was assessed using the MTT assay; c-myc expression was monitored by northern analysis, and DNA synthesis inhibition was assessed by monitoring the incorporation of labeled precursor into acid-precipitable DNA. Data for growth inhibition and DNA synthesis inhibition were taken from Ref. 2.

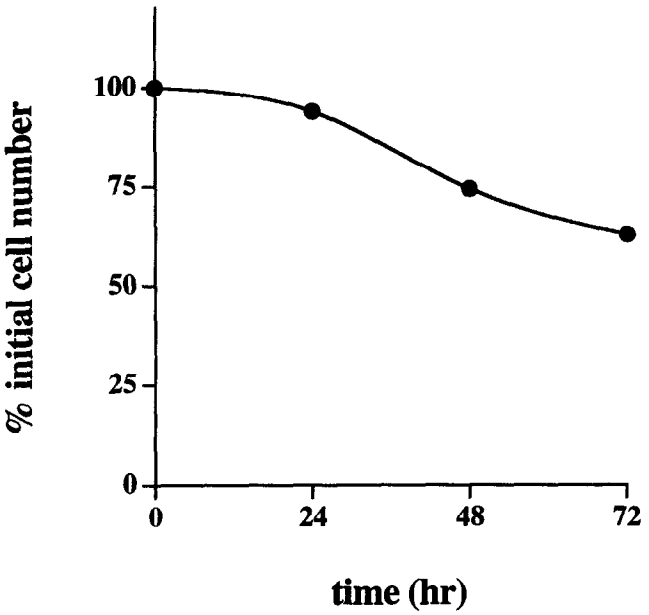

FIG. 4. Time-dependent reduction in number of viable cells after acute exposure to doxorubicin. MCF-7 cells in log growth were exposed to 1 μM doxorubicin for 4 hr. Cells were washed and resuspended in drug-free medium. Viable cell number was assessed at the indicated times based on trypan blue exclusion. Data are the means ± SEM for 5 separate experiments. The initial number of cells in assay varied between 10 and 20 × 10^6.

growth-stimulatory effect of doxorubicin,albeit at subtoxic concentrations. We propose that the subsequent decline in c-*myc* expression may reflect its function in a signal transduction pathway leading to growth arrest. This hypothesis is based on the close correlation between the concentration-dependent effects of the topoisomerase II inhibitors VM-26 and m-AMSA (and now doxorubicin) on c-*myc* expression and on growth inhibition [13, 14]. In recent work, we have observed that a similar correspondence exists with respect to suppression of c-*myc* expression and growth arrest by ionizing radiation in MCF-7 breast tumor cells.* While these correlations do not establish that growth arrest is dependent on the suppression of c-*myc* expression, the close relationship suggests that c-*myc* is somehow linked to the growth regulatory pathway.

As shown in this report and in our previous studies [13, 14], reduced c-*myc* expression in MCF-7 breast tumor cells occurs in response to drugs that produce predominantly G_2 arrest, whereas c-*myc* induction is generally associated with the G_0 to G_1/S transition [37]. However, a careful analysis of the cell cycle distribution data presented in Table 1 indicates that doxorubicin may be producing arrest in *both* G_1 and G_2, since a significant proportion of the cell population (between 54 and 58%) remained in G_1 at 24 and 48 hr after exposure of the cells to doxorubicin. In contrast, a classic G_2M block would result in a marked decline in the proportion of cells in the G_1 population. Interpretation of the data in Table 1 as indicating the induction of growth arrest in G_1 as well as G_2 would also be consistent with the inhibition of DNA synthesis by doxorubicin.

Various DNA-damaging agents that produce both G_1 and G_2 arrest have been reported to up-regulate the p53 tumor suppressor protein [38–40], and recent reports have suggested a relationship between p53 and the G_2 transition as well [41, 42]. It therefore appears possible that, like p53, c-*myc* may be involved in both the G_1 and G_2 checkpoints responding to DNA damage in the MCF-7 breast tumor cell line. In this regard, a role for c-*myc* in transition through G_2 has been reported by other investigators [43, 44]. One possibility currently under investigation is that an upstream signal, perhaps mediated via p53, leads to down-regulation of c-*myc* expression; this hypothesis is supported by the report that p53 has the capacity to transrepress c-*myc* [45].

The expression of c-*myc* has also been associated with DNA replication [46, 47], although this issue is not fully resolved [48, 49]. The close correlation between suppression of c-*myc* expression and inhibition of DNA synthesis by doxorubicin, as well as by VM-26 and m-AMSA [13, 14], supports such a relationship. However, because these effects occur within 2 hr of drug exposure, it is difficult to determine whether the suppression of c-*myc* expression precedes or follows the inhibition of DNA synthesis.

Non-apoptotic Cell Death in MCF-7 Breast Tumor Cells

The concentration of doxorubicin (1 μM) used in the present studies is clinically relevant in that this level is

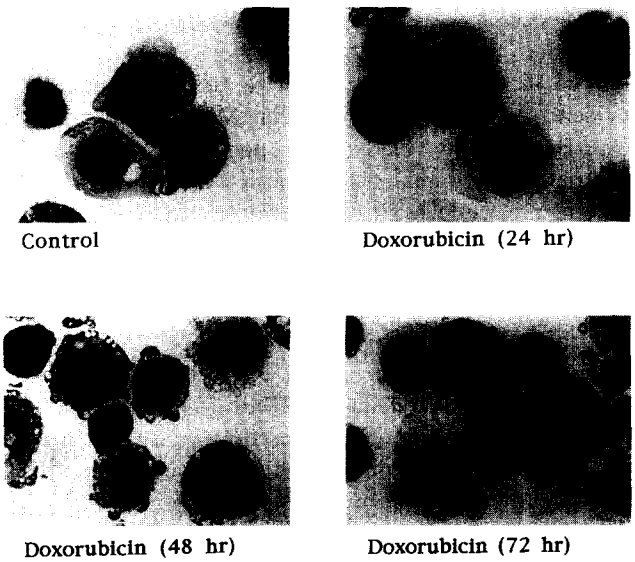

FIG. 5. Analysis of cell morphology. MCF-7 cells in log growth were exposed to 1 μM doxorubicin for 4 hr. Cells were washed and resuspended in drug-free medium. Cell morphology was assessed at the indicated intervals after cytospin and staining.

* Watson NC, Orr MS, Fornari FA and Gewirtz DA, Manuscript in preparation.

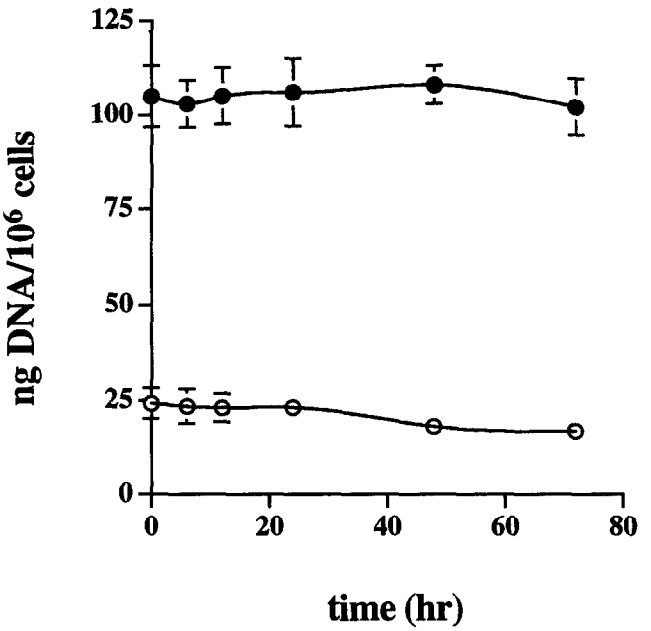

FIG. 6. Analysis of DNA fragmentation. MCF-7 cells in log growth were exposed to 1 μM doxorubicin for 4 hr. Cells were washed and resuspended in drug-free medium. DNA fragmentation was assessed quantitatively both in adherent (●) and nonadherent (○) cells. Data are the means ± SEM for 7 separate experiments.

achieved in the circulation within the first few hours after i.v. bolus administration [2, 3]. Acute exposure to 1 μM doxorubicin results in both growth arrest and cell death. In our previous studies in which MCF-7 cells were *chronically* exposed to 50 nM doxorubicin, we failed to detect either morphologic or biochemical evidence of apoptotic cell death [5]. In the previous as well as the present studies with MCF-7 cells after *acute* exposure to 1 μM doxorubicin, the cells appear to undergo autocatalysis, a process that does not reflect the different types of apoptotic cell death described in the studies by Zakeri *et al.* [50]. Thus, while evidence has been presented that MCF-7 cells have the capacity to undergo DNA fragmentation and apoptosis [51–55], our findings appear to be consistent with the report by Oberhammer *et al.* [32] demonstrating the relative refractoriness of MCF-7 breast tumor cells to apoptotic cell death.

The basis for the refractoriness of MCF-7 cells to the induction of apoptosis by, for example, doxorubicin remains obscure. One possibility is that down-regulation of c-*myc* expression may be incompatible with induction of p53 dependent apoptosis [56, 57]. Alternatively, various intrinsic cytoprotective mechanisms may be enhanced in MCF-7 cells. Recent studies have suggested a high ratio of Bcl-2 to Bax proteins in these cells [54, 58] and that Bcl-x_s sensitizes MCF-7 cells to chemotherapy-induced apoptosis [59]. Other mechanisms that could protect MCF-7 cells from programmed cell death include as yet undefined tyrosine kinases, as demonstrated in the case of the erythroleukemic K562 cell line [60], high endogenous protein kinase C activity [61], or matrix adherence [62]. It is possible, as demonstrated in a recent paper [59], that approaches which enhance apoptotic cell death in this breast tumor cell line may also sensitize these cells to chemotherapeutic agents.

This work was supported by grants from the Thomas M. Jeffress and Kate Jeffress Memorial Trust, Adria Laboratories, Grant CA-63753 from the National Cancer Institute, and Cancer Center Support Core CA-16059 from NCI. F.A.F. was supported by National Institute for Environmental Health and Safety Training Grant ESO-7087 from NIEHS and is the recipient of National Research Service Award HL-09241 from NHLBI. W.D.J. is the recipient of National Research Service Award CA-09380 from the National Cancer Institute.

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