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Permanent Cell Cycle Arrest in Asynchronously Proliferating Normal Human Fibroblasts Treated with Doxorubicin or Etoposide But Not Camptothecin

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ABSTRACT. Damage to DNA has been implicated in the induction of permanent cell cycle arrest or premature senescence in normal human fibroblasts. We tested the ability of a group of cancer chemotherapeutic agents or related compounds, which can cause DNA double-strand breaks (DSBs) directly or indirectly, to induce a permanent cell cycle arrest in normal proliferating fibroblasts. A brief treatment with etoposide, doxorubicin, cisplatin, or phleomycin D1 induced a block to S phase entry sustained through 15 days. Lower levels of these drugs did not induce appreciable levels of transient cell cycle arrest. Higher concentrations caused cell death that lacked the DNA degradation characteristic of apoptosis. Camptothecin, an agent that causes DNA single-strand breaks, which are converted to DSBs during S phase, was able to induce an efficient, but only transient, cell cycle arrest in these normal cells. The cells did not enter S phase until after removal of the camptothecin. These findings support the idea that permanent cell cycle arrest and cell death are typical reactions of these normal cells to drugs that can cause DSBs. In addition, we report data consistent with the concept that both actinomycin D and doxorubicin are sequestered by cells and slowly released in active form. This is consistent with the observation that both these drugs bind reversibly to intracellular components. BIOCHEM PHARMACOL **58**;4:675–685, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cell cycle; fibroblasts; doxorubicin; camptothecin; etoposide; cisplatin

Treatment of normal human fibroblasts with γ-radiation, a DNA strand-breaking agent, causes DNA damage and a long-term cell cycle arrest mediated by the tumor suppressor p53 [1–3]. These cells are blocked mostly in the G_1 and, to a lesser extent, the G_2 phase of the cell cycle and no longer can be stimulated to proliferate, although they remain viable. γ-Irradiation induces DNA damage including DSBs§ and SSBs at a ratio of about 1:10. In these normal cells, DNA DSB repair seems to be at a reduced rate compared with that in transformed cells [4, 5]. Also in normal fibroblasts, evidence suggests that even a single unrepaired DSB can result in a permanent cell cycle arrest [1, 4], and that permanent cell cycle arrest is the normal response of these cells to even relatively low levels of γ-irradiation.

It is not clear which cells, in addition to fibroblasts, undergo a long-term block to proliferation after DNA damage, although a long-term arrest has been reported to follow γ-irradiation of pigmented retinal epithelial cells [6]. Other primary cells, such as B cells and thyroid epithelium, which do not respond to damage by undergoing apoptosis, are also candidates to undergo a long-term cell cycle arrest [7–11].

Unlike normal human fibroblasts, cultures of immortal cell lines that express functional p53 rarely exhibit a long-term cell cycle arrest following γ-irradiation, but rather enter a transient G_1 arrest or die by apoptosis [12] (data not shown). DNA strand breaks seem to play an important role in this response [13]. Whether the cells stop proliferating or die depends upon a number of factors [14], such as the mutation or inactivation of various cell cycle regulatory proteins and oncoproteins (for example, the retinoblastoma protein), the presence of mitogens, the cell lineage, and the type of damage and its severity.

The response of cells to DNA damage is important, because it can serve as a defense mechanism against tumorigenesis in multicellular organisms [12]. DNA damage can lead to genomic instability and eventually to deregulated growth control [1, 15, 16]. Unless the damage can be repaired, it is in the best interest of the organism to have cells with DNA damage eliminated or blocked from proliferating. The response to DNA damage is also important because many anticancer agents exert their effects by causing DNA damage. The difference in response to DNA

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§ *Abbreviations*: DSB, double-strand break; SSB, single-strand break; p21, $p21^{waf1/cip1}$; cdk4, cyclin dependent kinase 4; and TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling.

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damage between normal and tumor cells may explain the selectivity of these agents for the tumor cells [12].

Like γ-irradiation, many cancer chemotherapeutic agents cause DNA DSBs, lesions that can be toxic to normal cells [16–18]. Doxorubicin, actinomycin D, and etoposide are topoisomerase II poisons that directly cause the formation of DNA DSBs and SSBs, where the DNA ends are bound reversibly to the topoisomerase enzyme. The SSBs can be converted to DSBs during DNA replication and RNA synthesis [18–20]. The ratio of DSBs to SSBs induced has been estimated to be from 1:1 to 10:1 [21, 22]. Actinomycin D also inhibits topoisomerase I directly, causing SSBs (and at higher concentrations can inhibit RNA synthesis) [20, 23]. Phleomycin D1, a member of the bleomycin family, is expected to cause DSBs and SSBs at a ratio of about 20:1, directly through the production of free oxygen radicals [24, 25]. Cisplatin and camptothecin cause DSBs indirectly. Cisplatin causes DNA chain cross-links that can be converted through the repair process to DSBs [18, 26]. Camptothecin is a topoisomerase I poison and causes protein-associated SSBs, which can be converted to DSBs when DNA is replicated in S phase [18–20, 27, 28]. Therefore, efficient formation of DNA DSBs by camptothecin is dependent on the traverse of S phase. DNA DSBs caused by different agents are not equal; studies in cell lines indicate that some breaks are more mutagenic than others [15]. It is thought that the exact nature of the break determines how efficiently it is repaired and how accurately [16].

It is not clear whether normal fibroblasts can respond to chemotherapeutic drugs by undergoing a long-term cell cycle arrest and/or whether the treated cells can efficiently recover from a cell cycle arrest induced by low levels of these agents. Past studies on these drugs and cells almost exclusively used colony formation assays, which do not differentiate cell death from a permanent block to proliferation. We have set out to characterize the response of normal asynchronous human fibroblasts to a brief treatment with six different DNA damaging agents. Four of these drugs, cisplatin, phleomycin D1, doxorubicin, and etoposide, can cause a permanent cell cycle arrest resembling premature cellular senescence across the whole cell population. Cisplatin and phleomycin D1 can induce this block to proliferation with minimal toxicity. Camptothecin appears only to cause a transient arrest in the majority of the treated cells, whereas the response to actinomycin D is concentration dependent. We also show that doxorubicin, and likely actinomycin D, can be sequestered by cells and then slowly released in active form.

MATERIALS AND METHODS

Drugs

Actinomycin D, etoposide, and cisplatin were from the Sigma Chemical Co. Camptothecin stocks for duplicate experiments were from Sigma and Arcos. Doxorubicin and phleomycin D1 (Zeocin) were from Fujisawa U.S.A., Inc. and Invitrogen, respectively. Stock solutions of cisplatin and phleomycin were dissolved in PBS; actinomycin D was dissolved in ethanol, and all were stored at −70°. Dilution of these and doxorubicin (which was stored at 4°) was in PBS. Camptothecin and etoposide were stored and diluted in DMSO.

Cell Culture and Drug Treatment

HDF-3 human foreskin fibroblasts, between population doublings 20 and 30, were grown under 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Invitrogen). Cells were plated at approximately 10^3 cells/cm^2 as indicated, 48 hr before the addition of drug in most experiments. Unless otherwise noted, drugs were left on the cells for 12 hr, and then the cells were washed twice with PBS and fed fresh medium. For all camptothecin and etoposide treatments, the final concentration of DMSO in the medium was 0.5% (which alone had no effect on the S phase index).

To produce conditioned medium, after drug treatment the cells were washed with PBS, and then fresh growth medium was added. After 36 hr (or less if indicated), this medium was "conditioned." To assay cell cycle inhibition, the conditioned medium was transferred to asynchronously growing cells. After 36 hr, [^{3}H]thymidine was added to 1 μCi/mL, and the cells were incubated for another 24 hr before fixation with glutaraldehyde and autoradiography.

RNase Protection Assay

Total RNA was prepared using guanidinium isothiocyanate extraction. The production of stable RNA was scored using internally ^{32}P-labeled antisense RNA probes. Hybridizations to cellular RNA followed by T2 digestion and separation of protected products on denaturing PAGE were as described earlier [29]. The template for the p21 probe contains nucleotides 1–322 from the p21 cDNA [30]. The cyclophilin probe was obtained from Ambion and gives a protected fragment of 103 bases.

Western Blotting

Cell lysates were prepared and characterized essentially as described [29]. Protein concentrations were determined by a modified method of Bradford (Bio-Rad), and 50 μg was loaded per lane onto a 12% SDS–PAGE gel. Protein was electrotransferred to nitrocellulose (Schleicher & Schuell), followed by staining with Ponceau S to verify transfer and equal loading. Detection was performed using an enhanced chemiluminescence detection system (ECL, Amersham) according to the manufacturer's recommendations. Mouse monoclonal antibodies, anti-p53 Ab-6 (DO-1) and anti-p21 Ab-1, were purchased from Oncogene Research Products.

Toxicity and Cell Death Assays

Cell number was determined by counting cells in ten random 10x fields for each sample after fixing, which amounted to approximately 1000 cells for control samples. The counts from at least three experiments were used in the determination of the mean and SEM. Note that the level of trypan blue staining in preliminary experiments was insignificant even following the most toxic treatments, indicating that adherent cells were viable.

To detect nuclear condensation and fragmentation 24 hr after treatment, cells were fixed, stained with 0.1 μg/mL of Hoechst 33258, and viewed under a fluorescence microscope. The TUNEL assay was performed with an *In Situ* Cell Death Detection Kit essentially as described by the manufacturer's recommendation (Boehringer Mannheim) [31]. Samples were examined for fluorescein labeling by fluorescence microscopy.

RESULTS

Cancer chemotherapeutic drugs and related compounds cause DNA damage that induces a transient cell cycle arrest or apoptosis in immortal cell lines. We studied the effect of these drugs on normal fibroblasts, focusing on the recently elucidated phenomenon of permanent cell cycle arrest, which is a form of premature cellular senescence [1, 29, 32]. Six different drugs were used at concentrations such that the highest levels caused some cells to die and the lowest levels caused some cells to undergo a cell cycle arrest. We routinely used 12-hr treatments; this length of exposure is grossly similar to exposures to these drugs in cancer chemotherapy [26, 32–36]. Cells were asynchronously proliferating at the initiation of treatment. There are four possible outcomes to this treatment: a short- or long-term cell cycle arrest, cell death, or no detectable response. Assays were performed on total cell populations (not clonal assays) in order to distinguish long-term cell cycle inhibition from cell death.

Permanent Blocks to the Cell Cycle after Transient Treatment with Drugs That Cause DNA DSBs

Brief treatments with sufficient levels of actinomycin D, etoposide, cisplatin, doxorubicin, and phleomycin D1 induced a permanent cell cycle arrest in normal human fibroblasts. Cell proliferation of viable cells after drug treatment was determined by [^{3}H]thymidine incorporation, which identifies cells traversing S-phase and can be differentiated from DNA repair, which results in much lower levels of incorporation [37]. Following a 12-hr drug treatment, the cells were washed twice with PBS and given fresh medium. [^{3}H]Thymidine in fresh medium was added 1, 4, and 15 days after the drug was removed and was left on for 24 hr before the cells were fixed and prepared for autoradiography. Day 15 plates also received fresh medium at day 7. The proliferation rates after the different treatments were calculated (Fig. 1). Treatments that gave labeling indices higher than 60% often resulted in partially confluent cells, so the exact S phase index is not shown. By day 15 uninhibited cells were confluent, and these are marked as such. At the higher concentrations of all drugs (except camptothecin), virtually the entire cell population could be blocked from entering S phase between 1 and 4 days after treatment. Cultures that were completely blocked 1 day after treatment with doxorubicin, etoposide, phleomycin D1, and cisplatin remained blocked by day 4. In contrast, cultures initially blocked from entering S phase after treatment with 0.04 μg/mL of actinomycin D showed significant labeling by day 4. This indicates that a large fraction of the cells treated with actinomycin D were only temporarily blocked from entering S phase.

In this study, long-term cell cycle arrest is said to occur when 15 days after treatment, less than 10% of a cell population traverses S phase in a 24-hr period. Very few cells treated with the higher concentrations of doxorubicin, etoposide, phleomycin D1, and cisplatin entered S phase at this time, indicative of a long-term cell cycle arrest. There was an increase in labeling at day 15 for intermediate concentrations of all the drugs. This does not necessarily indicate a transient cell cycle arrest but was more likely due to a few cells that never stopped proliferating and that eventually outgrew the growth-arrested cells. With a doubling time of approximately 1 day, if just 1% of the cells continued to proliferate, by day 15 they would dominate in number.

Survival after Drug Treatment

To determine if cell cycle arrest was the favored response versus cell death, we measured viable cell counts after treatment with concentrations of drugs that cause a long-term cell cycle arrest. For cisplatin and phleomycin D1, the complete population could be blocked from entering S phase with little, if any, cell loss (Table 1). There was a great variability among the different drugs in their ability to cause a long-term cell cycle arrest and to kill cells. Doxorubicin and etoposide caused a complete cell cycle arrest, but roughly half the cells slowly died off by day 4 and even more by day 15. At the highest concentration of actinomycin D the extant cells did not enter S phase, but only about 11% had survived treatment at day 15. In summary, a major response to treatments with cisplatin, phleomycin D1, doxorubicin, and etoposide was a long-term cell cycle arrest, although at high enough levels of drugs, cell death occurred. It was not clear if the cell death that occurred was apoptosis, as there was no evidence for increased rates of chromosome condensation, with the one exception of camptothecin-treated cells (data not shown). Also, none of the drug treatments led to increased DNA fragmentation as measured by the TUNEL assay (data not shown), although control serum-deprived mouse NIH3T3 cells showed 5% TUNEL-positive cells [38].

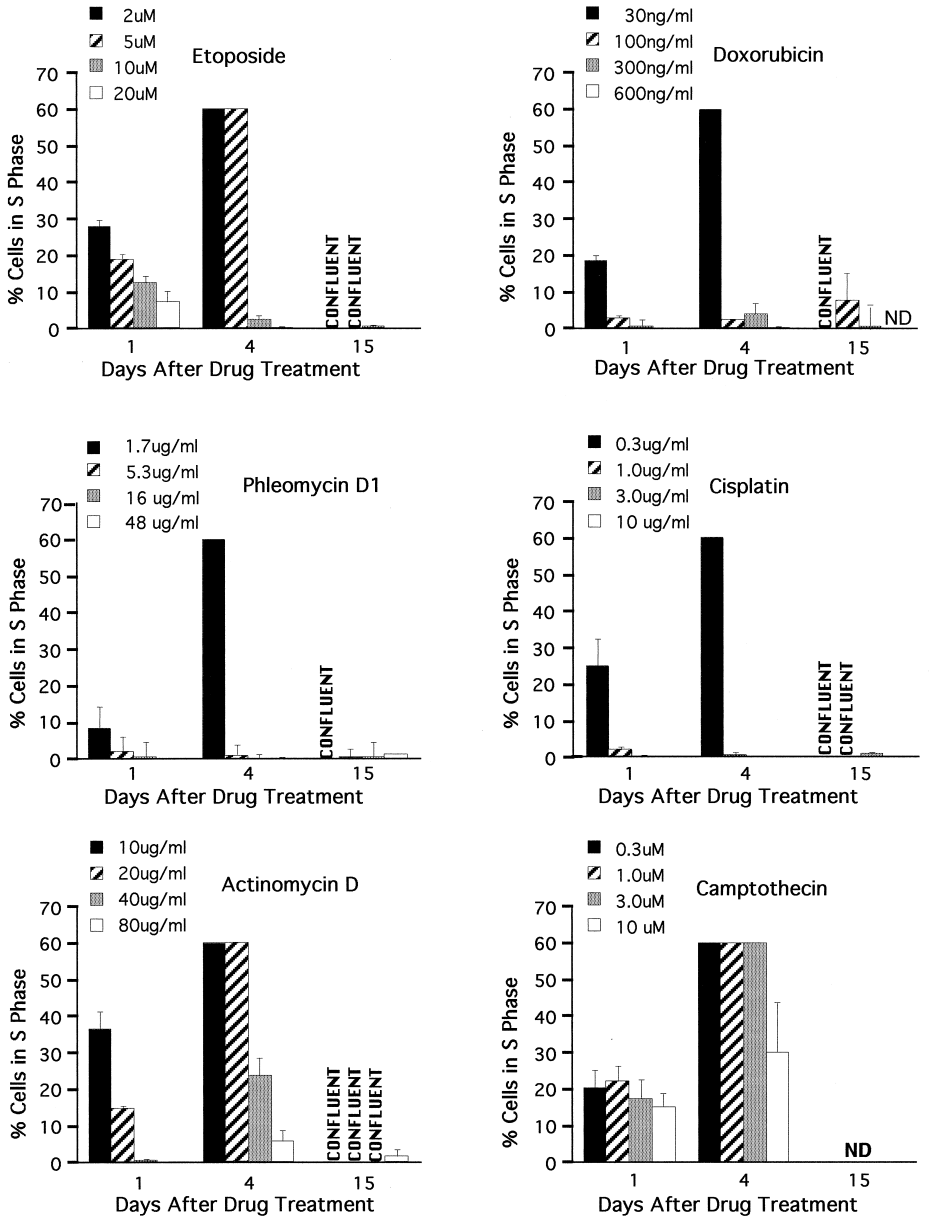


FIG. 1. Cell cycle block over long times after treatment of normal human fibroblasts with DNA damaging drugs. Asynchronous HDF-3 fibroblasts ($1.1 \times 10^3/cm^2$) were treated with each drug at four different concentrations for 12 hr. The cells then were washed, and at various times after the completion of the drug treatment, 1 μCi/mL of [^{3}H]thymidine was added for 24 hr. Cells were fixed and processed for autoradiography. Cells with darkly labeled nuclei were counted as S phase cells. Treatments that gave labeling indices higher than 60% showed areas of confluent cells, so exact counts are not shown. Fully confluent wells also are indicated. All experiments were done four times in duplicate, and the SEM is indicated. Control untreated cells showed a mean labeling index of 83.8 ± 1.8%, with over 800 cells counted.

Induction of a Transient Cell Cycle Arrest by Camptothecin

It long has been thought that chemotherapeutic drugs cause a transient cell cycle arrest in normal cells and that these cells resume proliferation after they recover [12]. Whereas we have demonstrated that doxorubicin, etoposide, phleomycin D1, and cisplatin were capable of causing a long-term cell cycle arrest in normal fibroblasts, it was unclear whether these drugs can induce a transient cell cycle arrest. To make that determination, we measured the reduction in numbers of cells in S phase during the last hour of treatment with each drug, when we expected reversible effects on cell proliferation to be reaching peak levels. We then compared this with the relative number of cells that traverse S phase during the 12 hr following drug washout. Cells plated 4 days earlier were treated with fresh medium alone or with drug (Fig. 2). The level of S phase traverse (normalized for the level in untreated cells) before and after drug removal was determined. Only the cells treated with 0.3 μM camptothecin (a drug that induces just SSBs in the

TABLE 1. Survival of fibroblasts after drug treatment that induces a long-term cell cycle arrest

Treatment	Survival (%)		
	Day 1	Day 4	Day 15
Cisplatin (3 μg/mL)	100 ± 4.0	100 ± 4.0	98 ± 4.0
Phleomycin D1 (265 μ/mL)	100 ± 9.2	103 ± 3.5	62 ± 5.2
Actinomycin D (80 ng/mL)	28 ± 8.3	20 ± 7.0	11 ± 1.5
Etoposide (10 uM)	100 ± 7.8	79 ± 9.3	35 ± 8.9
Doxorubicin (100 ng/mL)	87 ± 6.5	52 ± 7.5	42 ± 18

Cells ($1.1 \times 10^3/cm^2$) were treated for 12 hr with drugs at levels that cause a sustained cell cycle arrest. Shown are the normalized cell counts at the three time points after treatment. Each point is the mean ± SEM of four independent experiments. Values are normalized to untreated control cells at time 0. In total, an area of the plate containing 800 cells in the untreated plates was counted for each assay point.

majority of cells) showed a distinct increase in the normalized level of cells in S phase in the 12-hr period after drug removal. At this drug concentration, the reentry into the cell cycle was quite rapid.

These experiments did not rule out the possibility that the other drugs induced a true transient cell cycle arrest in a small minority of the cells not detected by the assay. It is also important to note that earlier work, showing that cisplatin-induced cross-links are repaired efficiently, does not contradict our findings. Those experiments showed that an efficient recovery from cisplatin-induced DNA damage required that the cells be held in an extended G_0 arrest [39, 40].

Paradoxical Increase of Camptothecin Toxicity at Lower Drug Concentrations

Because of the inability of camptothecin in our experiments to cause a long-term cell cycle arrest, we tested similar concentrations, but with longer incubation times, to give the drug more time to evoke an effect. This approach elicited a more efficient cell cycle arrest in the period 24–48 hr after drug removal (Fig. 3A). The percentage of S phase cells was very low after exposure to the two lower camptothecin concentrations, and at the two higher concentrations we never observed any cells traversing S phase. Curiously, by 4 days after drug removal the level of proliferation and the number of viable cells had become inversely proportional to the level of drugs used. Specifically, cells treated with 1 μM camptothecin had resumed proliferating, whereas 90% of the cells tested with 0.3 μM camptothecin were still inhibited from entering S phase. This phenomenon was observed in four independent experiments. By day 4, the cells treated with 1 μM camptothecin were double in number versus the cells treated with 0.3 μM camptothecin (Fig. 3B). This unexpected result may be explained by the fact that high concentrations of drug are better at blocking S phase entry (Figs. 2 and 3). Because cells are particularly vulnerable to incurring DNA DSBs during S phase, this may act as a protecting force against irreversible DNA damage. With high concentrations of drug, the cells would not enter S phase until after drug removal.

TABLE 2. Cell cycle inhibition by medium conditioned by cells treated previously with actinomycin D or doxorubicin

Medium is from cells treated with:	Percentage of S phase cells after medium addition
Mock	71 ± 5.8
Bleomycin (0.06 U/mL)	63 ± 8.7
Actinomycin D (0.04 μg/mL)	0 ± 0.1
Cisplatin (1 μg/mL)	67 ± 3.0
Etoposide (20 μM)	63 ± 6.1
Doxorubicin (200 ng/mL)	17 ± 16

Conditioned medium was from cultures treated with levels of drugs that caused greater than 95% cell cycle block. Asynchronously proliferating cells were treated with conditioned medium for 36 h and then were incubated with 1 μCi/mL of [^{3}H] thymidine to allow the identification of cells transiting S phase. All tests were done 3 times. For each treatment 600 cells were counted; after mock treatment 426 were positive. Means ± SD are shown.

Cell Cycle Inhibitory Activity in Medium from Actinomycin D- and Doxorubicin-Treated Cells

In preliminary trials we found that the feeding schedule of some of the drug-treated cells could influence the results greatly. Specifically, we found that an additional medium change 2 days after actinomycin D or doxorubicin treatment would reduce the number of cells that remained blocked from entering S phase at later time points. This suggested that either the replenishment of growth factors in the medium had a stimulatory effect on proliferation, or there was some agent in the spent medium that was growth-inhibitory. To test these possibilities, medium from cultures treated with these drugs was transferred to plates containing asynchronously growing fibroblasts. To maximize the sensitivity of this assay, the conditioned medium was left on the cells for 36 hr, and then [^{3}H]thymidine was added for a 24-hr period. As a control, medium from untreated cultures of asynchronous cells of approximately the same density was used. Medium conditioned by cells treated with either actinomycin D or doxorubicin was capable of arresting nearly 100% of the cells, whereas medium conditioned by cells treated with the other drugs had no significant inhibitory effect relative to the control (Table 2). Identical experiments with the same drug treatments but from plates lacking cells showed no effect (data not shown). In fact, the more cells on the plate exposed to doxorubicin or actinomycin D, the higher the levels of inhibitory activity in the conditioned medium (data not shown). This probably caused the variability in the inhibitory activity that was released from doxorubicin-treated cells (Table 2). These findings suggest that some cell cycle

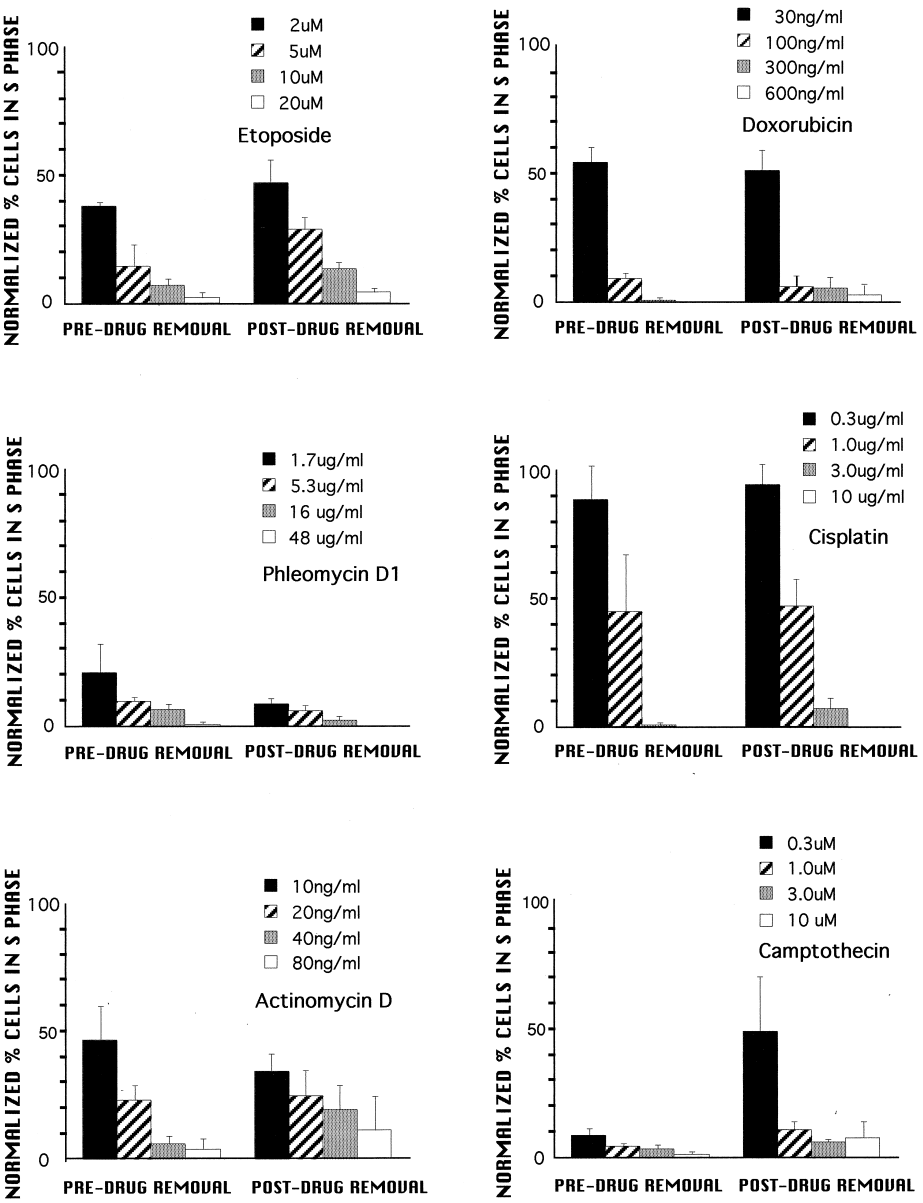


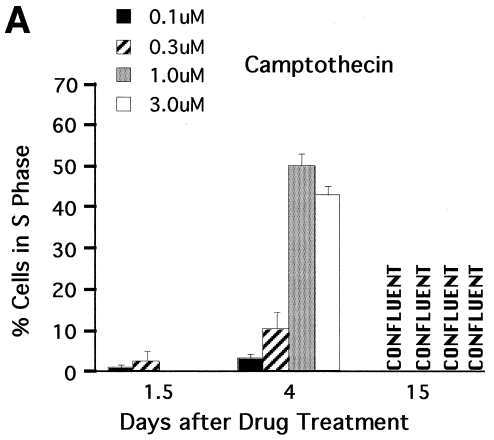
FIG. 2. Rapid cell cycle reentry after camptothecin removal. HDF-3 cells (0.5×10^3) were plated 4 days prior to the assay. Half the cells were labeled with [^{3}H]thymidine (10 μCi/mL) in the last 1.5 hr of the drug treatment and then fixed. In the remaining plates, immediately after drug removal, [^{3}H]thymidine was added at 2 μCi/mL for a 12-hr period. Percentages of cells in S phase were normalized to that produced in mock-treated cells. For the early labeling period this was 82 ± 8.7% for 400 cells counted, and for the second labeling period it was 87 ± 5.7% for 430 cells counted. All experiments were done at least 3 times, and error bars indicate the SD.

inhibitor was being released from the cells or cellular matrix.

The release of the inhibitory activity from doxorubicin- or actinomycin D-treated cells was not rapid. The bulk of the inhibitory activity from doxorubicin-treated cells was released from cells by 12 hr after removal of the drug (Fig. 4). Cells treated with actinomycin D showed a similar time course, with the bulk of the activity released later than 10 min after washing the cells but earlier than 12 hr after treatment (data not shown). The fact that doxorubicin and actinomycin D have been shown to build up in cells at high levels [33, 34, 41–43] suggested that for both of these drugs the inhibitor might be the drugs themselves.

Induction of p53 and p21 by Medium from Actinomycin D-Treated Cells

Cells treated with agents that cause DNA damage show induction of the tumor suppressor p53 [44]. The rise in p53 protein levels and its activation result in an increase in transcription of several genes including the cyclin-dependent kinase inhibitor p21 [30, 45]. By contrast, there is little evidence that cellular growth inhibitory factors work by increasing p53 levels in fibroblasts. To determine if the cell cycle arrest induced by the conditioned medium caused p53 induction, protein extracts and total RNA were prepared from cells fed with medium conditioned by cells previously



B

	CELL NUMBER	
Treatment	Day 1	Day 4
0.1uM	65.6±6.5	101±13
0.3uM	62.1±4.8	97.8±5.7
1.0uM	52.1±9.2	197±52
3.0uM	11.4±3.0	129±17

FIG. 3. Concentration-dependent effects of 36-hr camptothecin treatment. (A) Cell cycle transit. Experiments were conducted as described in the legend to Fig. 1 except that the duration of drug treatment was 36 hr. The experiment was repeated 5 times; error bars represent SEM. Mock-treated cells (no drug) showed an S phase index of 86 ± 2.7% (344 out of 400 cells). (B) Effects on cell number. Data are expressed as relative cell number after treatment, normalized to 100 initially. The experiment was done 5 times; error bars represent SEM.

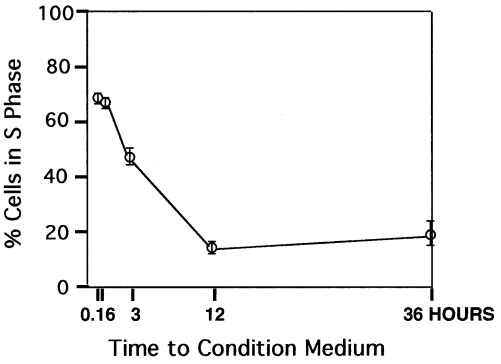


FIG. 4. Time course for release of inhibitory activity from doxorubicin-treated cells. To create conditioned medium, a set of 3.5-cm plates with 0.5 × 10^6 cells were treated with 600 ng/mL of doxorubicin in 5 mL of fresh growth medium for 12 hr. Cells then were washed twice, and new medium was added. At the times indicated, conditioned medium was removed from one plate, and the plate was discarded. To test the inhibitory activity of medium conditioned for various times, medium was added at a final dilution of 1 to 5 to asynchronous cultures of HDF-3 cells. Shown is the mean S phase index with the standard deviation for cells treated with medium conditioned for different times. The experiment was done twice, and each value represents at least 3 trials. Cells treated with medium from non-drug-treated cells showed a mean S phase index of 71 ± 3.2%, with over 465 cells counted.

treated with actinomycin D, and as a negative control the phleomycin D1-related compound bleomycin. Protein samples were subjected to SDS–PAGE and immunoblotting. As a positive control, extracts and RNA also were prepared from cells treated directly with this drug. An RNase protection assay (Fig. 5A) showed that the conditioned medium from actinomycin-treated cells resulted in an induction of p21 mRNA similar to that caused by the direct application of the drug. In addition, p53 and p21 proteins also clearly were induced in the cells given the conditioned medium from actinomycin-treated cells and control drug-treated cells (Fig. 5B). This is most compatible with a model where the drug itself is released in active form from the actinomycin D-treated cells.

Sequestration and Release of Doxorubicin by Cells

For doxorubicin, we wanted to determine more directly if the drug is sequestered by the cells and later released, in the same or altered form, or if the inhibitory activity in the conditioned medium is actually due to some factor synthesized by the cell. To rule out these latter possibilities, we estimated the inhibitory activity of medium conditioned by doxorubicin-treated cells relative to the drug itself in the biological assay and compared it to the drug concentration determined by using solid phase extraction and HPLC with fluorescence detection [46].

Fibroblasts were fed various dilutions of medium conditioned by doxorubicin-treated cells and then assayed by [^{3}H]thymidine labeling 36 hr later. Another similar titra-

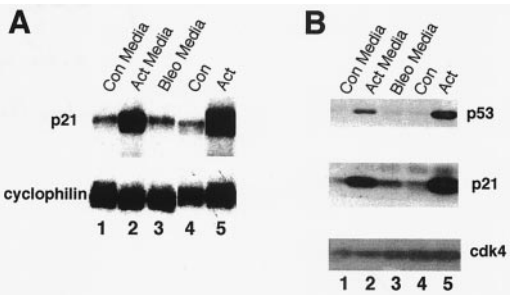


FIG. 5. Increases in p21 mRNA and protein levels induced by medium conditioned by cells treated with actinomycin D. Asynchronously growing HDF-3 cells were exposed to medium conditioned by control cells (lane 1), actinomycin D-treated cells (lane 2), or bleomycin-treated cells (lane 3) for 36 hr (see Materials and Methods). As further controls, cells were untreated (lane 4), or treated directly with 0.04 μg/mL of actinomycin D for 12 hr (lane 5), then washed, fed, and incubated for an additional 24 hr before harvesting. (A) Shown is an autoradiograph of the gel from an RNase protection analysis performed to quantitate p21 mRNA levels. Cyclophilin mRNA was measured as an internal control. Lanes are identified as above. (B) Western analysis on extracts prepared from cells treated as above. The blots were probed with antibody as labeled: anti-p53, anti-p21, and as a control anti-cdk4.

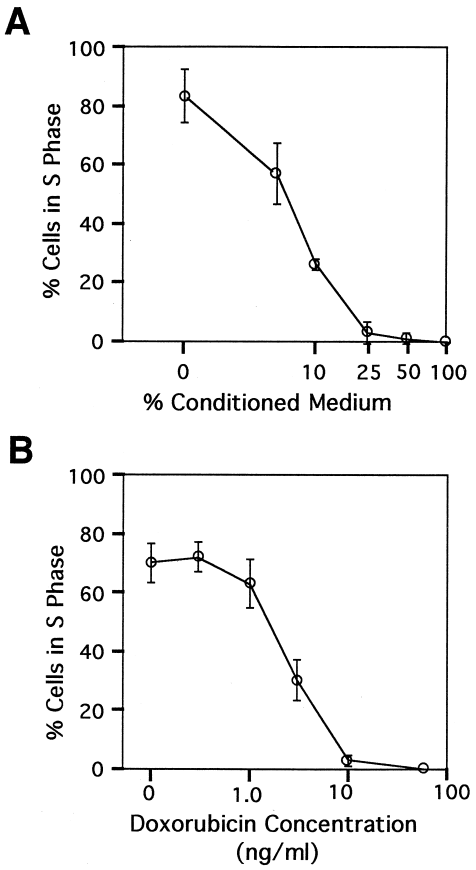


FIG. 6. Titration of the inhibition of the G_1/S phase transition by conditioned medium from doxorubicin-treated cells and by doxorubicin directly. Asynchronous fibroblasts were exposed for 36 hr to conditioned medium from cells treated with doxorubicin (A), or exposed to the drug directly (B). Shown is the percentage of cells that passed though S phase in the time period 36 through 60 hr after treatment with the various dilutions of medium or drug. The values are the means of three independent experiments for each titration ± SD. The labeling index of untreated control cells for the conditioned medium titration was 84 ± 9.1%, whereas for the drug titration it was 68 ± 6.7%. Over 400 cells were counted for each value.

tion was performed directly with the drug doxorubicin (Fig. 6). By comparing the IC_{50} of the medium and drug, we estimated that the doxorubicin concentration in the medium was approximately 35 ng/mL. The doxorubicin concentrations found in conditioned medium by HPLC analysis in two separate experiments were 11.1 and 16.2 ng/mL. The similarity of the values arrived at by the two different assays suggests that doxorubicin is retained by the cells or cellular matrix after washing and then released still in active form.

DISCUSSION

We have shown that a brief treatment with doxorubicin, etoposide, phleomycin D1, cisplatin, and actinomycin D, drugs that directly or indirectly induce DNA DSBs, caused a permanent cell cycle arrest in normal human fibroblasts. In our experiments with asynchronous cells, these drugs tended to have an all or nothing effect on cell proliferation. Low levels of doxorubicin, etoposide, phleomycin D1, and cisplatin all were incapable of inducing a detectable transient cell cycle arrest in a mass culture. This by no means indicates that low levels of these drugs do not produce DNA damage [47], only that there was no detectable block to S phase entry. In addition to DNA DSBs, these drugs produce DNA SSBs, or in the case of cisplatin, high levels of DNA adducts including cross-links. Both of these types of damage can be repaired in these cells, although efficient repair of cross-links takes days and requires that the cells be maintained in a nonproliferative state by contact inhibition [39, 40].

Our results indicate that under the conditions used, the lowest levels of drug sufficient to block cell cycle transit across a population of cells produce enough of the right kind of damage (complex lesions such as DNA DSBs) so that the majority of cells do not reenter the cell cycle. Although these drugs have various other toxic effects [26, 33], the common feature is the induction of DNA DSBs, corroborating the hypothesis that this type of lesion (or other complex DNA damage) is the critical one in regard to permanent cell cycle arrest [1, 4]. Etoposide-, doxorubicin-, cisplatin-, and phleomycin D1-treated cells all show elevated expression of a marker of cellular senescence, the lysosomal SA-β-galactosidase, by 9 days after treatment [29, 48] (data not shown). This would imply that the agents that cause DNA DSBs induce a senescence-like state, as is the case for bleomycin-treated cells [29].

Camptothecin-induced cell cycle arrest can be short-lived. Camptothecin treatment of asynchronously proliferating fibroblasts for 12 hr causes an efficient cell cycle arrest (92%), which to a large degree is reversed rapidly upon drug removal. Camptothecin is a topoisomerase I poison and causes SSBs, which can be converted to DSBs when the replication fork passes through unrepaired damage [20, 27, 28, 49]. Camptothecin also inhibits RNA synthesis. DNA DSB formation by camptothecin has a strict requirement for S phase [20, 27]. One reason why transient cell cycle arrest was observed with camptothecin could be the repair of the single-strand breaks before DNA replication. There is a total block to S phase entry in the presence of high concentrations of drug, so that except for the cells that are in S phase at the initiation of treatment, little, if any, of the damage can be converted to a toxic form. This does not seem to be the case with at least some other cells that have been tested. For example, extended incubations of camptothecin, or its derivatives, with immortal mammary cell lines showed prolonged cell cycle arrest or cell death. It took 9–10 days for cultures to resume growth after drug removal, suggesting that the vast majority of the cells had been blocked irreversibly from proliferating [50]. It is possible that the DNA damage checkpoints that respond to SSBs produced by camptothecin in this established cell line may not be as tightly regulated as in normal human fibroblasts.

A final piece of evidence for the existence of a strict

checkpoint control block after camptothecin treatment is in an apparent paradox. Right after treatment for 36 hr with 1 μM camptothecin there was a complete S phase block, whereas treatment with 0.1 μM of the drug allowed very low but detectable numbers of cells to enter S phase (see Fig. 3). By 4 days after drug removal the effect was reversed; cells treated with lower amounts of drugs actually showed much higher levels of S phase inhibition. These data are compatible with the idea that at low levels of camptothecin a small percentage of cells continue to enter S phase. We speculate that as new damage occurs in these cells it will be converted to DNA DSBs and that the cells may become permanently cell cycle arrested. It also suggests the idea that high versus low level treatment with camptothecin as a chemotherapeutic agent may at times be less toxic to normal fibroblasts and perhaps other normal cells in the body.

Actinomycin D is another drug that is capable of causing a temporary cell cycle arrest. This may be due to the fact that in addition to poisoning topoisomerase II and inducing DSBs directly, this drug also alters topoisomerase I activity [20]. In addition, as has been described earlier, actinomycin D at concentrations much higher then those used in this study inhibits total DNA and RNA synthesis in a cell [29]. Possibly low levels of this or another activity [34] result in a reversible arrest. The fact that the reversibility is quite slow is probably due to the fact that the drug is retained in cells even after the cells are washed.

We have found that both doxorubicin and very likely actinomycin D are retained in or on cells in an excretable form. This was not true for the other drugs tested even though they caused similar or higher levels of cell cycle arrest and toxicity (Table 2 and data not shown). It has been shown previously that actinomycin D intercalates into DNA and accumulates in cells, whereas doxorubicin can build up in cells due to the pH differential between the intra- and extracellular environment [41–43]. Our results extend earlier findings to indicate that these drugs can be released in active form from normal fibroblasts at concentrations high enough to block S phase entry in untreated cells. Because the release of inhibitory activity (Fig. 4) peaked 3–12 hr after the drug washout (when little or no cell death was observed), it suggests that the drugs were released from live cells rather than dead cells. This work may have implications for clinical treatment in that the sequestering effect can be expected to increase the length of exposure of tissue to these drugs, as evidenced by their relatively long plasma half-life [51, 52], which in turn can increase both the effectiveness and toxicity of these drugs.

Cell survival after induction of a permanent cell cycle arrest is drug dependent. Survival ranged from 100% with 3 μg/mL of cisplatin to only 11% after 0.4 μg/mL of actinomycin D treatment. Drug treatments that killed cells did not induce two of the hallmarks of apoptosis, DNA degradation or chromosome condensation, with the exception of camptothecin, which induced chromosome condensation. It remains unclear whether these cells treated with high levels of DNA damaging agents (or γ-irradiation) [53] die through apoptosis or necrosis. It should be noted that normal human fibroblasts induced to die by lysosomal rupture show DNA degradation, indicating that they are capable of undergoing a typical apoptotic cascade [54].

If asynchronous fibroblasts are a representative model for cells of the body, it is apparent that recovery of cells from drug toxicity severe enough to block cell proliferation often does not occur. Work with only two drugs (actinomycin D and camptothecin) showed clearly that a majority of cells were able to return to the cell cycle after treatment with enough drug to induce a cell cycle arrest. To some degree, drugs such as phleomycin D1, etoposide, doxorubicin, and cisplatin must rely on other properties to allow them to target tumor cells while sparing fibroblasts and similar cells [25, 26, 33–36]. Differences in drug uptake, turnover, and molecular target concentrations between tumor and normal cells appear to be responsible for tumor cell killing.

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