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Transforming Growth Factor-β3 Protection of Epithelial Cells from Cycle-Selective Chemotherapy *In Vitro*

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ABSTRACT. The transforming growth factor-β (TGF-β) family of regulatory growth factors can reversibly arrest cell division in the G_1 phase of the cell cycle. Previously, TGF-β3 was shown to protect epithelial cells and hematopoietic cells from cytotoxic damage *in vitro* and *in vivo*, and to reduce the severity and duration of oral mucositis induced by 5-fluorouracil (5-FU) *in vivo*. In the present study, we tested whether TGF-β3 can protect epithelial cells from a range of chemotherapy drugs with differing mechanisms of action, using the CCL64 cell line as a model system. We report that preincubation of cells with TGF-β3 for 24 hr resulted in enhanced clonogenicity following exposure to vinblastine, vincristine, etoposide, taxol, ara-C, methotrexate, or 5-FU. Protection was measured in colony-forming assays, which demonstrated that the protected cells could re-enter the cell cycle and undergo multiple rounds of cell division. At high cytotoxic drug concentrations, absolute colony counts were increased for the cultures prearrested by TGF-β3, as compared with the proliferating control cultures. The effects of TGF-β3 were reduced for cisplatin and doxorubicin, drugs that are toxic to cells throughout the cell cycle. Thus, TGF-β3 can effectively reduce the cytotoxicity of anticancer drugs that act predominantly in S or M phase of the cell cycle. BIOCHEM PHARMACOL 53;8:1149–1159, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. TGF-β3; chemoprotection; epithelial cells; cell cycle, chemotherapy

Negative regulators of cell growth have attracted attention in experimental strategies aimed at decreasing the adverse effects of chemotherapy and radiation on normal proliferating tissues. A reversible arrest in one of the gap (G_1, G_2) phases of the cell cycle, for example, might increase the survival of cells exposed to drugs that target DNA synthesis or mitosis. Recently, the multifunctional cytokine interleukin-11 was shown to arrest the growth of a cell line derived from intestinal epithelium [1], and to protect stem cells in the small-intestinal crypts of irradiated mice [2]. Similarly, the TGF-βs† comprise a class of growth inhibitory factors active on bone marrow and epithelial cells [3–5]. Of the three human TGF-βs (β1, β2 and β3) that have been characterized and cloned, TGF-β1 has been the best studied with regard to its effects on the cell cycle. Key intracellular targets for TGF-β1 inhibitory action are molecules that regulate G_1-to-S phase progression, such as c-*myc* [reviewed in Ref. 5], a subset of the CDKs, and their cognate cyclins [6, 7]. Depending on the cell line studied, TGF-β1 has been shown to negatively regulate S phase entry at several levels, including inhibition of CDK4 and CDK2 protein synthesis [8, 9], inhibition of cyclin A and E gene transcription [9], and induction of one or more CDK inhibitor proteins, such as $p15^{INK4b}$ [10], $p21^{WAF-1}$ [11] and $p27^{KIP-1}$ [12]. The end result of TGF-β1 signaling is decreased c-*myc* expression and an inhibition of Rb phosphorylation, leading to arrest of cells in G_1 [13, 14].

Several observations suggest that the TGF-βs could prove to be useful in protecting major tissues from the adverse effects of many chemotherapeutic drugs. First, the TGF-βs inhibit the proliferation of hematopoietic [15, 16] and epithelial [1, 2, 17, 18] cells, two lineages in humans which are affected adversely by DNA damage in the bone marrow, the intestine, the oral mucosa, the gonads, the hair follicles, and other organs [19, 20]. Second, most cells that are arrested in G_1 by TGF-β do not exit the cell cycle permanently, and can reestablish cell division (after a lag phase) once TGF-β is removed from the medium [21]. Third, many human cancers are unable to arrest their growth in response to TGF-β, either due to inactivation or mutation of the TGF-β receptors themselves [22], or due to defects in the ability to regulate S phase entry [23–28]. The finding that many tumor lines continue to grow in the

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† *Abbreviations*: TGF-β, transforming growth factor β; 5-FU, 5-fluorouracil; MTX, methotrexate; CDK, cyclin-dependent kinase; ATCC, American Type Culture Collection; BrdU, bromodeoxyuridine; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; and SRB, sulforhodamine B.

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presence of TGF-βs is consistent with this expectation [29–31].

Several groups have examined the ability of one or more TGF-βs to enhance the survival of different cell types exposed to anticancer agents [15, 31–34]. All of the human TGF-βs studied have chemoprotected cell lines from one or more drugs and, in some studies, have increased the proportion of mice surviving exposure to lethal doses of the same agents [32–34]. For example, the administration of either TGF-β1 or TGF-β2 protected murine bone marrow progenitor cells from 5-FU *in vitro*, increased the proportion of mice surviving 5-FU injection, and potentiated hematopoietic recovery following bone marrow transplantation [32]. Studies with human cells have focused on the bone marrow compartment [15, 31], a major site of toxicity for many chemotherapy regimens [20]. Both TGF-β1 and TGF-β3 were shown to increase the cycling time of primary erythroid and myelomonocytic progenitor cells and, accordingly, TGF-β3 was found to protect a $CD34^+$ enriched population of bone marrow cells from 4-hydroperoxycyclophosphamide (4-HC) *in vitro* [31]. In contrast, TGF-β3 failed to confer protection to several leukemia cell lines tested in the same assays [31].

As previous reports of chemoprotection mediated by the TGF-βs have been based on single chemotherapeutic agents [15, 31–34], a goal of this study was to compare the ability of TGF-β3 to protect epithelial cells from a panel of cytotoxic agents with differing mechanisms of action. Agents with specific effects on DNA synthesis (ara-C) or mitosis (*Vinca* alkaloids, taxol) were studied, as were agents that act throughout the cell cycle via multiple mechanisms (doxorubicin, cisplatin). First, the concentration and timing of TGF-β3 pre-exposure were optimized to protect a test cell line (CCL64 mink epithelial cells) during subsequent drug exposure. Second, TGF-β3-mediated protection was assessed predominantly by colony formation, a long-term assay in which viable cells are required to undergo multiple cell divisions to be scored. Here, we report that incubation of CCL64 cells with TGF-β3 for 24 hr before exposure to vinblastine, vincristine, etoposide, taxol, ara-C, MTX, and 5-FU, but not doxorubicin or cisplatin, increased the number of surviving cells retaining colony-forming ability.

MATERIALS AND METHODS

Cells and Recombinant Human TGF-β3

Mink lung epithelial cells (CCL64) were obtained from the ATCC, and cultured in Dulbecco's modified Eagle's medium (DMEM) or, for experiments with MTX, modified Eagle's medium alpha (MEM-α) supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Cell doubling time was determined by seeding exponentially dividing CCL64 cells into 96-well microtiter plates at 5000 cells/well in 200 μL medium. At different time points thereafter, 50 μL of a 0.2% solution of MTT (Sigma, St. Louis, MO) [35] in PBS was added directly to the medium of triplicate wells, and the plates were returned to 37° for 2 hr. The medium was aspirated, and the metabolized MTT formazan product was solubilized with 100 μL of ethanol/acetone (50%/50%). Plates were read at 540 nm in a microplate colorimeter; the mean background absorbance from triplicate wells lacking MTT was subtracted, and net absorbance curves were plotted. From the curves, the doubling time of CCL64 cells was found to be 16–18 hr. Biologically active human TGF-β3 was expressed in CHO cells, or in *Escherichia coli* as previously described [36]. Aliquots of TGF-β3 were stored at −20°, thawed only once, and used on the same day.

DNA Synthesis (BrdU) Assays and Kinetics of the TGF-β3 Growth Arrest

DNA synthesis, as measured by BrdU incorporation, was determined using a commercial immunocytochemical proliferation kit (Amersham, Arlington Heights, IL) under conditions specified by the vendor. Briefly, exponentially dividing CCL64 cells were seeded into microtiter plates at a density of 5000 cells/well. After the addition of TGF-β3 and/or chemotherapy drugs in triplicate wells (see below), DMEM containing BrdU labeling reagent was added to give a final 1:500 dilution of the BrdU stock. Cells were labeled for 2 hr at 37°, washed in PBS, and fixed in acetic acid/ethanol (5%/95%) for 30 min at room temperature. Subsequent steps were also performed at room temperature. Fixed cells were washed four times in PBS containing 0.1% Tween-20 (PBS-Tween), and blocked in 3% BSA/PBS-Tween (100 μL/well) for 15 min. Reconstituted anti-BrdU detection reagent (50 μL/well) was added for 1 hr, plates were washed with PBS-Tween as before, and diluted peroxidase-conjugated anti-mouse IgG (50 μL/well) was added for 30 min. Plates were washed four times with PBS-Tween, and peroxidase substrate solution was added (100 μL/well). Color development was arrested with stop solution, and the absorbance of the plate was measured at 405 nm. Mean background absorbance from triplicate wells lacking BrdU was subtracted, and net BrdU incorporation was determined.

To measure the duration of TGF-β3 exposure needed to arrest the asynchronous growth of CCL64 cells, microtiter plates were seeded at 5000 cells/well. The following day, the medium was replaced with medium containing 15 pM TGF-β3 (~10 ng/mL). Cells were then processed for BrdU incorporation immediately, or at 2, 4, 8, and 27 hr post TGF-β3 addition. The data were plotted as the net absorbance (absorbance reading minus background from wells without BrdU) on the *y*-axis versus time on the *x*-axis. To determine whether TGF-β3 was required continuously to achieve growth arrest, cells were seeded into microtiter plates as above. The following day, the culture medium was replaced with medium containing 15 pM TGF-β3. At different time points thereafter, TGF-β3 was washed out of the medium. Twenty-five hours after the addition of factor, all of the plates were processed for BrdU incorporation.

Results were plotted as net absorbance on the y-axis versus duration of TGF-β3 exposure on the *x*-axis.

Cytotoxic Concentration–Response Measurements

The anticancer agents included were: cytosine β-D-arabinofuranoside hydrochloride (ara-C), 5-FU, MTX, vinblastine (sulfate salt), vincristine (sulfate salt), etoposide, taxol, doxorubicin (Adriamycin®), and cisplatin. The drugs were resuspended in 100% DMSO at 3–25 mg/mL, divided into aliquots, and stored at −70° until used, except for cisplatin, which was freshly dissolved in DMSO prior to each experiment. All compounds were purchased from Sigma except for taxol (ICN, Costa Mesa, CA). For the generation of concentration–response curves, cells were seeded into microtiter plates (2000/well) in 100 μL of medium/well. Three hours later, 2-fold serial dilutions of the drugs in medium were added to give a final volume of 200 μL/well. Each drug concentration was tested in triplicate wells. Cells were exposed to the drugs for 24 hr, followed by washing the monolayers with PBS, addition of fresh medium, and incubation of the plates at 37° for an additional 48–72 hr. MTT assays were performed as described above. Net absorbance values for each drug concentration were plotted as a percentage of mean net absorbance for control wells without drug. From the concentration–response curves, five or more concentrations of each drug were selected for the TGF-β3 chemoprotection assays.

Cell Growth and Clonogenic Assays

Chemoprotection assays were performed in microtiter plates using SRB [37] staining as a measure of cell number. Cells were pretreated with 0, 2, 20, or 200 pM TGF-β3 for 3 days, with the inclusion of ara-C on days 2 and 3, followed by SRB staining.

For colony assays, subconfluent CCL64 cells in log-phase growth were seeded into 6-well plates, at a density of 50,000 cells/well in a volume of 3 mL. For each typical experiment (one drug at five concentrations), two plates were seeded. Three hours after plating, the majority of cells had adhered. Medium was removed, and replaced with fresh medium containing 15 pM TGF-β3 (six wells), or control medium without TGF-β3 (six wells). Plates were then returned to 37° for 24 hr, to allow the TGF-β3-treated cells to become growth arrested. Then the medium was replaced with fresh medium with or without TGF-β3 as before, now containing one of the selected drug concentrations. The sixth well served as the DMSO-only control. The plates were again returned to 37° for 24 hr. Cells were washed, trypsinized, and split into six 10-cm dishes into medium lacking factor or drug. Several dilutions were made (e.g. 1:50, 1:250, 1:750), so that accurate extrapolations to the fraction of clonogenic cells could be made. Plates were incubated for an additional 10–12 days, and colonies were stained with MTT and enumerated. The statistical significance of TGF-β3 treatment was characterized by ANOVA.

All figures below present the data from the colony and SRB assays in two formats. The first format presents data points as percentage values, relative to the control cells that received neither TGF-β3 nor drug. This method illustrates the inhibition of cell growth caused by TGF-β3, and how the arrest translates into more colony-forming cells following exposure to high concentrations of drugs. In the second method, data from the TGF-β3-treated and -untreated wells were analyzed separately, so that separate IC_{50} values could be calculated for each drug studied. As summarized in Table 1, the second method illustrates the degree to which TGF-β3 increases the IC_{50} for different drugs.

RESULTS

The strategy for using growth inhibitors as *in vitro* chemoprotecting agents has been to transiently arrest the growth of cells just prior to and during drug exposure [2, 15, 31–34]. Here, experiments using TGF-β3 were designed to achieve complete growth arrest before drug addition, yet allow rapid reentry of any surviving cells into the cell cycle. Maximal inhibition of DNA synthesis, as assayed by BrdU incorporation, occurred when CCL64 cells were exposed to TGF-β3 for 24 hr or longer (Fig. 1A). Maximal inhibition required the continuous presence of TGF-β3 during this period, with shorter exposures (ranging from 10 min to 8 hr, Fig. 1B), or pulsed 30-min exposures every 3 or 6 hr (data not shown) resulting in partial inhibition. The arrested CCL64 cells were viable and, by BrdU analysis, could reenter S phase 24 hr after removal of TGF-β3 (not shown).

TGF-β3-Mediated Protection from the Antimetabolites ara-C, MTX, and 5-FU

We first tested whether growth arrest mediated by TGF-β3 could protect CCL64 cells from ara-C, a drug that is selectively toxic for cells in S phase ([33], reviewed in Ref. 38). Figure 2 shows the results of a short-term microtiter assay in which adherent cells were incubated in the absence or presence of TGF-β3 (three concentrations) for 3 days, with ara-C (or the DMSO control) also present on days 2 and 3. Results from wells receiving DMSO alone indicated that 3 continuous days of TGF-β3 exposure reduced the SRB signal by about 60%, consistent with the doubling time of CCL64 cells (20 hr), the kinetics of the growth arrest, and the ability of SRB to stain both dividing and nondividing cells. As shown in Fig. 2A, cultures arrested by TGF-β3 appeared to be resistant to decreases in SRB staining caused by ara-C, whereas the proliferating cultures were clearly sensitive to the drug, exhibiting an IC_{50} of 6 μg/mL. At concentrations of ara-C 28 μg/mL or higher, TGF-β3-pretreatment translated into a slight absolute increase in SRB staining levels, as compared with the proliferating cells. All drug IC_{50} values were increased to > 278 μg/mL (> 1 mM) for the cells pretreated with TGF-β3 (Fig. 2B). Statistical analysis of the normalized data (Fig. 2B) indicated that all TGF-β3 groups were significantly different

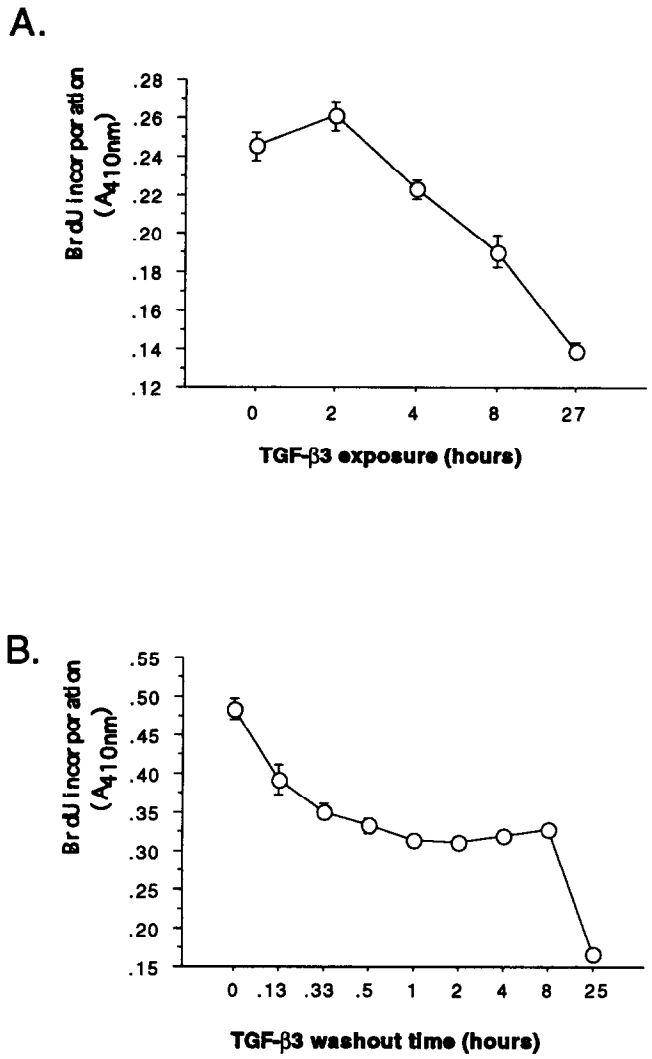

FIG. 1. Effect of TGF-β3 exposure time on BrdU incorporation by CCL64 cells. (A) Cells in microtiter plates were incubated with 15 pM TGF-β3 for the times indicated, and processed immediately afterward for BrdU incorporation. Each point is the mean of six replicates from one experiment where error bars indicate the standard errors of the means. (B) Requirement for continuous factor exposure. Cells in microtiter plates were incubated with 15 pM TGF-β3 for the indicated times, then washed, and refed with fresh medium lacking factor. All wells were then processed after the 25-hr timepoint for BrdU incorporation. Each point is the mean of twelve replicates from one experiment where error bars indicate the standard errors of the means.

from the no TGF-β3 control at all concentrations of ara-C tested (ANOVA, $P \leq 0.0001$, df = 5). Examination of the IC_{50} curves indicated that the 20 and 200 pM TGF-β3 concentrations resulted in higher staining than 2 pM (the reported IC_{50} TGF-β3 concentration for CCL64 cell growth inhibition [34, 36]). In all subsequent experiments, 15 pM TGF-β3 was used to achieve maximal chemoprotection.

The microtiter assay results in Fig. 2 suggested that TGF-β3 pretreatment could increase the IC_{50} of ara-C for CCL64 cells. However, such assays were usually terminated 3 days after cell plating, to prevent confluence and contact inhibition in the control wells from producing an overestimate of the proportion of cells in the test wells. To determine whether cells exposed to TGF-β3 and drug retained long-term growth potential, we developed an assay in which cultures were trypsinized and cells were replated following exposure to the cytotoxic agents. Figure 3 shows the results of such a colony-forming assay in which ara-C was again used as the test compound. The IC_{50} for ara-C in the colony-forming assay was calculated at 0.04 μg/mL (vs 6 μg/mL in the microtiter assay), supporting the notion that colony formation was a more stringent criteria for chemoprotection than SRB staining for cellularity. However, the conclusions regarding the effects of TGF-β3 were analogous to those obtained previously: in the absence of TGF-β3, ara-C reduced the number of colonies in a concentration–dependent manner. In the presence of TGF-β3, colony formation was reduced initially > 80% (compare no ara-C values in Fig. 3A). However, the TGF-β3-arrested cells retained significantly increased colony-forming potential at the 0.125, 0.25 and 25 μg/mL concentrations of ara-C (Fig. 3B; ANOVA: $P < 0.01$), with a shift in IC_{50} to > 25 μg/mL. At drug concentrations ≥ 1 μg/mL, the absolute colony counts were higher in the TGF-β3-treated cell population.

MTX, a folic acid analog, inhibits *de novo* purine and thymidylate biosynthesis, with resulting nucleotide depletion. The ability of MTX to induce both single- and double-stranded DNA breaks may also contribute to its cytotoxic effects [38, 39]. Therefore, experiments using MTX were conducted in MEM-α, which lacks ribonucleotides and deoxyribonucleotides. Concentrations of MTX from 0.05 to 300 μg/mL in unarrested cells resulted in marked colony inhibition with a calculated IC_{50} of < 0.05 mg/mL (Fig. 4A). In contrast, TGF-β3 arrest led to fewer colonies in the absence of drug, but more colonies for MTX concentrations > 0.5 μg/mL (Fig. 4A), and significant improvements in relative colony-forming efficiency for all MTX concentrations (Fig. 4B; ANOVA $P \leq 0.0001$; df = 5). Thus, as observed with ara-C, the TGF-β3 growth arrest could be exploited to increase the number of colony-forming cells after exposure to high concentrations of a cytotoxic drug.

The pyrimidine analog 5-FU exhibits multiple mechanisms of cytotoxicity which allow it to be active throughout the cell cycle, with highly proliferating cells the most sensitive [39]. Previously, we reported that TGF-β3 could decrease the incidence of ulcerative mucositis in hamsters treated systemically with 5-FU [34]. In CCL64 cells, TGF-β3 increased the IC_{50} value ~2-fold, from 0.5 to 1 μg/mL (Fig. 4, C and D). The IC_{50} shift was reproducible in three separate experiments, demonstrating that, *in vitro*, TGF-β3-arrested cells were weakly protected from this agent. When normalized to the control mean, TGF-β3 treatment was significantly different from control at the 0.25 and 1.0 μg/mL 5-FU concentrations (Fig. 4D; ANOVA $P = 0.03$ and $P = 0.006$, respectively; df = 5). Absolute colony numbers were higher for the unarrested cells, irrespective of the concentration of 5-FU tested (Fig. 4C).

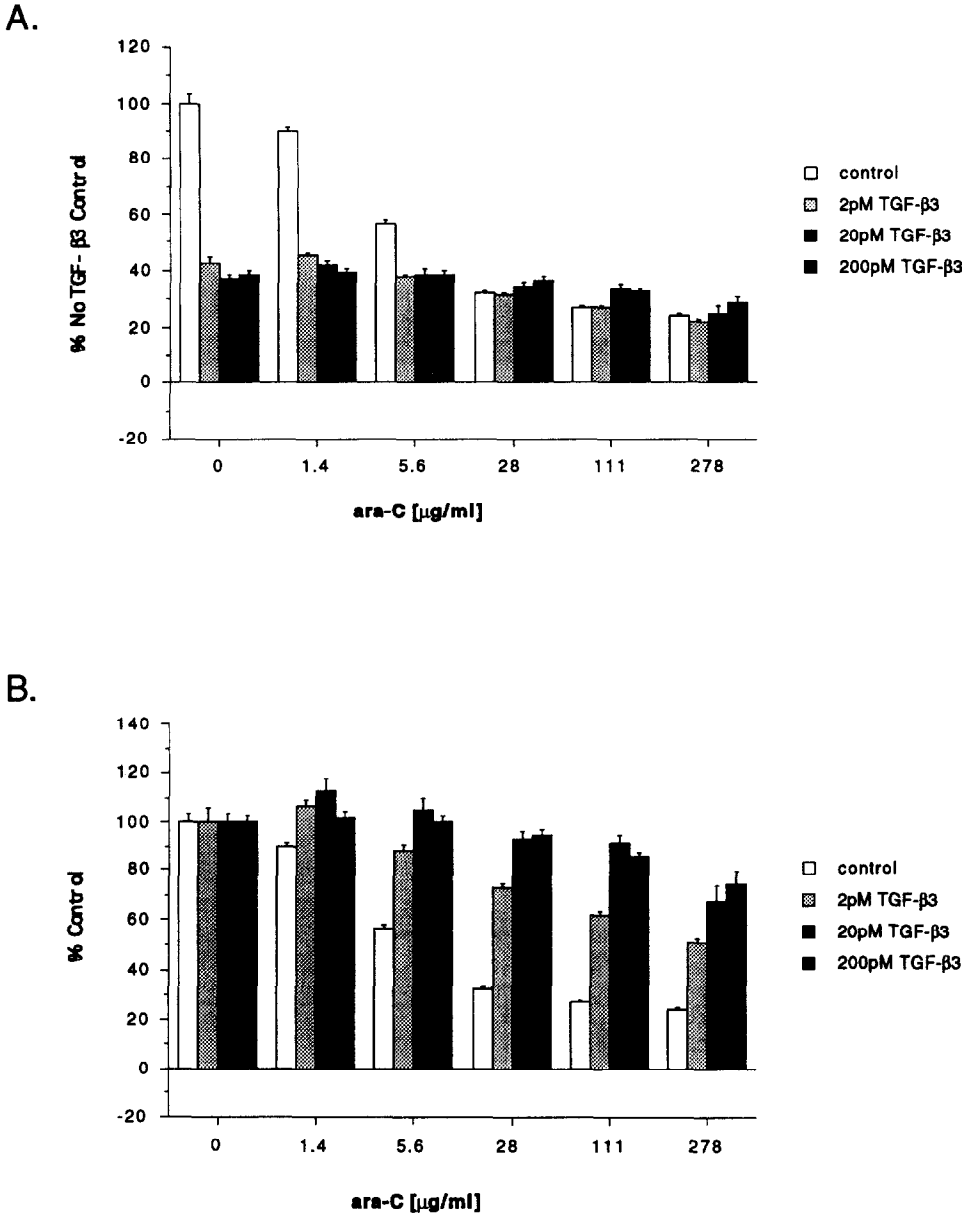

FIG. 2. TGF-β3 protection of CCL64 cells from ara-C-mediated toxicity. Cellularity was measured using the sulforhodamine B dye assay. Cells in microtiter plates were pretreated with medium containing 0, 2, 20, or 200 pM TGF-β3 for 24 hr, followed by a 48-hr exposure to ara-C (0–278 μg/mL) in medium containing the same concentrations of TGF-β3. Each point is the mean of sixteen replicates from one experiment, where error bars indicate the standard errors of the means. (A) All absorbances are shown as percent of control wells (those receiving neither TGF-β3 nor drug). (B) The same absorbance values from panel A were retabulated, with each pretreatment condition (0 vs 2, 20, and 200 pM TGF-β3) analyzed separately, and each of the no-drug (DMSO only) control values set to 100%.

Protection of CCL64 Cells from the Mitotic Inhibitors Etoposide, Taxol, Vinblastine, and Vincristine

Etoposide (VP-16), an epipodophyllotoxin, is thought to exert its major cytotoxic effects by inducing DNA strand breaks subsequent to interactions with topoisomerase II [38]. In the colony assays, the IC_{50} value of etoposide for growth inhibition of CCL64 cells was 0.15 μg/mL (Fig. 5A). As expected, TGF-β3 reduced the population of colony-forming cells by 80%, but the remaining 20% fraction could withstand 24-hr etoposide exposures to 1 μg/mL. Absolute colony counts were greater for the TGF-β3-treated cells when exposed to etoposide concentrations of 0.5 to 1 μg/mL. TGF-β3 treatment was found to increase the IC_{50} 3-fold, from 0.15 to 0.45 μg/mL (Fig. 5B and Table 1). TGF-β3 treatment was significantly different from control at etoposide concentrations up to 1 μg/mL (Fig. 5B; ANOVA $P \leq 0.001$; df = 5).

The mitotic inhibitor taxol arrests cells in late G_2/M phase by enhancing tubulin polymerization with subsequent stabilization of microtubules [40]. Based on a calculated IC_{50} value for taxol of 2 ng/mL for CCL64 cells in log-phase growth, we chose a range of concentrations (1–100 ng/mL) for testing in the chemoprotection assay. TGF-β3 pretreatment resulted in a consistent chemoprotection from this drug, and increased the absolute colony counts over a broad range of taxol concentrations (5–10 ng/mL; Fig. 5C). In the TGF-β3-arrested cultures, colony formation plateaued at ~60% of no drug values; thus, an IC_{50} for the chemoprotected cells was not reached (Table 1). TGF-β3 treatment was significantly different from control at taxol concentrations above 1 ng/mL (Fig. 5D; ANOVA $P \leq 0.0001$; df = 5).

Binding of the *Vinca* alkaloids vinblastine and vincristine to tubulin results in metaphase arrest and cell lysis [38].

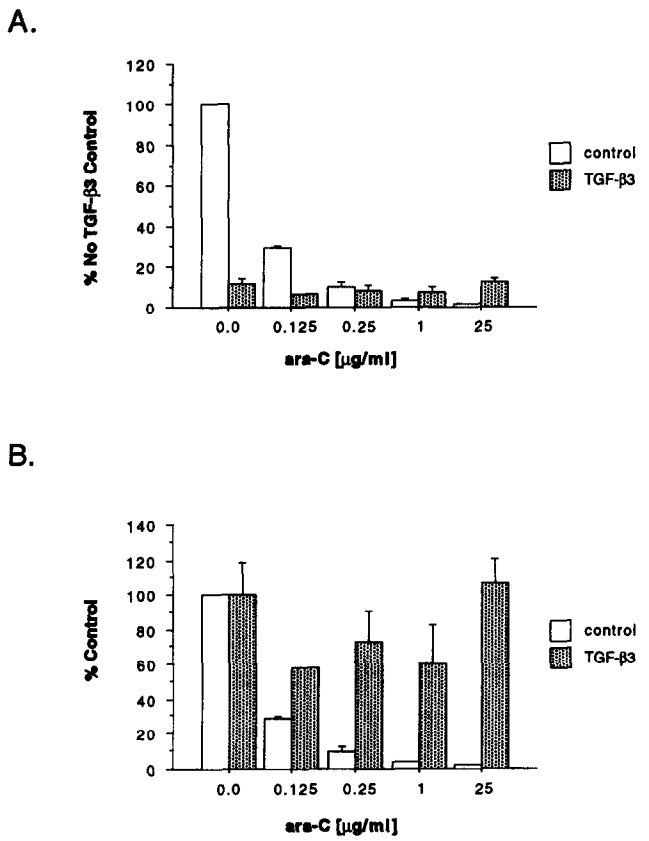

FIG. 3. TGF-β3 protection of CCL64 cells from ara-C-mediated cytotoxicity. Cell survival was measured using a clonogenic, colony-forming assay. Cells in 6-well plates were pretreated with medium containing 0 or 15 pM TGF-β3 for 24 hr, followed by a 24-hr exposure to ara-C (0–25 μg/mL) in medium with or without TGF-β3 as before. Cells were then washed, trypsinized, and seeded into 10-cm dishes for colony formation. Each point is the mean of six replicates from one experiment, where error bars indicate the standard errors of the means. (A) All colony counts are shown as a percentage of control wells that received neither TGF-β3 nor drug. (B) Analysis of the same data in panel A with the TGF-β3-treated and control populations treated separately, and all of the no-drug (DMSO only) control counts set to 100%.

Based on the concentration–response curves for vinblastine- and vincristine-mediated growth inhibition of CCL64 cells, concentrations of each drug were chosen to inhibit colony formation by 15–50% for the lowest concentration and 90–98% for the highest concentration. TGF-β3 addition resulted in a significant and absolute increase in CCL64 colony-forming potential for both vinblastine and vincristine at multiple concentrations (Fig. 5, E–H). The IC_{50} values were shifted > 100-fold for the arrested cells (Fig. 5, F and H), such that half-maximal inhibition was not achieved with any drug concentration tested (Table 1). Significant chemoprotection was observed for all concentrations of vinblastine (Fig. 5F; ANOVA $P \leq 0.01$; df = 6) and for vincristine concentrations of ≥ 5 μg/mL (Fig. 5H; ANOVA $P \leq 0.0001$; df = 5).

Results with Cisplatin and Doxorubicin

As shown in Figs. 2–5, TGF-β3-arrested cells were efficiently protected from agents with activities confined to S or M phase. We next tested two agents, cisplatin and doxorubicin, that are active throughout the cell cycle. For cisplatin (*cis*-diaminedichloroplatinum II), toxicity arises via multiple mechanisms, which may include the cross-linking of intracellular molecules. Cisplatin is active throughout the cell cycle, although it may be most toxic to cells in G_1 [38]. The IC_{50} of cisplatin for CCL64 cells was found to be 0.3 μg/mL (Fig. 6A). TGF-β3 had no statistically significant impact on colony-forming efficiencies, and the IC_{50} plots for arrested versus unarrested cells were virtually superimposable (Fig. 6B; ANOVA $P > 0.05$; df = 5). Lack of protection from cisplatin was not due to a loss of TGF-β3 activity, as shown by the typical decrease in colony numbers for the control no-drug wells.

Doxorubicin (Adriamycin®) is also toxic to cells throughout the cell cycle, via mechanisms that target topoisomerase II and cause DNA damage, with disruption of DNA replication and transcription [38]. Doxorubicin was a potent inhibitor of CCL64 colony formation, with an IC_{50} of 1.1 ng/mL (Fig. 6C). Although TGF-β3 clearly arrested cell division in the experiment, relative colony-forming efficiencies were not improved significantly (Fig. 6D). The slight IC_{50} increase (from 1.1 to 1.5 ng/mL; Table 1) was more than offset by the reduction in cell number mediated by TGF-β3. TGF-β3 treatment was significantly different from control at doxorubicin concentrations up to 2 ng/mL (Fig. 6D; ANOVA $P \leq 0.002$; df = 5).

DISCUSSION

The TGF-βs are potent and reversible growth inhibitors that arrest in the G_1 phase of the cell cycle [3–5, 15–18]. Since the majority of anticancer agents are most active on cycling cells, we reasoned that a transient TGF-β3 arrest might confer resistance to high-dose chemotherapy. In particular, drugs with S or M phase-specific mechanisms of action should be most susceptible to attenuation by the TGF-β3 block. Previously, we reported that TGF-β3 addition to CCL64 cells increased the number of cells surviving exposure to vinblastine [34]. Here we tested the ability of TGF-β3 to protect cells from a panel of agents, which included mitotic inhibitors (vinblastine, vincristine, taxol, etoposide), antimetabolites (ara-C, 5-FU, MTX), an antitumor antibiotic (doxorubicin), and a carboplatinum agent (cisplatin). As summarized in Table 1, TGF-β3 was found to decrease the sensitivity of cells to vinblastine, ara-C, MTX etoposide, and taxol. While TGF-β3 was weakly protective from 5-FU, little or no protection from doxorubicin or cisplatin was observed.

For etoposide and 5-FU, TGF-β3 increased the IC_{50} values without changing the overall negative slopes of the concentration–response curves. In contrast, for ara-C, vin-

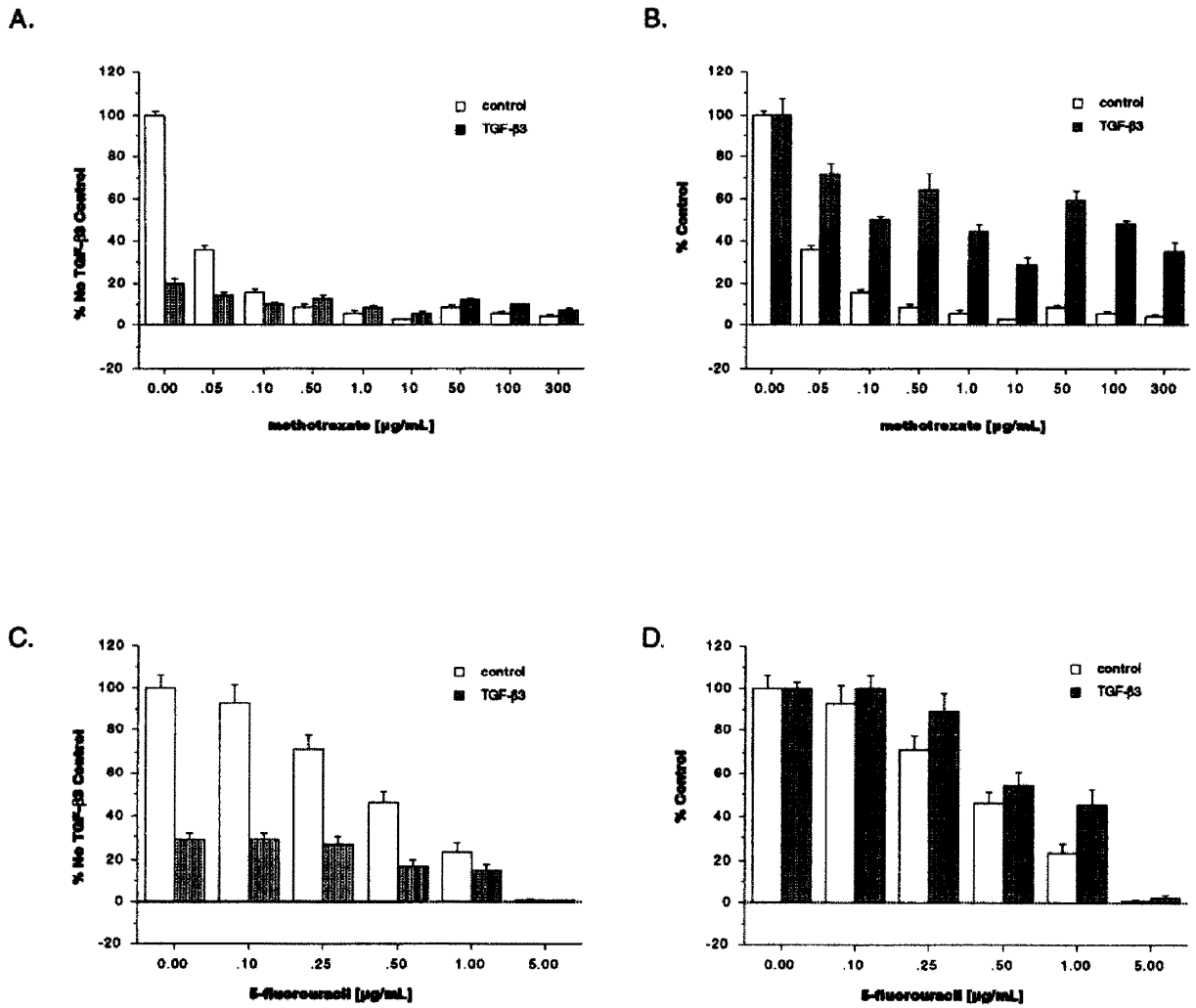

FIG. 4. Effects of TGF-β3 arrest on colony inhibition mediated by MTX and 5-FU. MTX (A and B) or 5-FU (C and D) was added to cultures of CCL64 cells, either in log-phase growth or pre-arrested with 15 pM TGF-β3 as before with ara-C (Fig. 3 and Materials and Methods). Colony counts were either tabulated as a percentage of the colonies formed from wells receiving neither TGF-β3 nor drug (A and C), or the TGF-β3-treated and control cell populations were analyzed separately, with each of the no-drug (DMSO only) control counts set to 100% (B and D). Each point is the mean of six replicates from one (MTX) or three (5-FU) experiments, where error bars indicate the standard errors of the means.

blastine and taxol, TGF-β3 appeared to limit the population of susceptible cells: once a maximum kill was reached, further increases in drug concentrations exerted no further cytotoxic effect within the range tested. The results suggest that a TGF-β-mediated reversible G_1 arrest is the predominant mechanism underlying chemoprotection *in vitro* and that TGF-β3 may be useful in the protection of epithelial cells from agents that are cytotoxic in S or M phases of the cell cycle. However, our studies do not preclude the possibility that TGF-β3 can be used to spare cells from cytotoxic agents that induce DNA damage in G_1, or to collaborate with intrinsic checkpoints to enhance the survival of cells with existing DNA damage. Radiation-induced checkpoints in G_1 and G_2, for example, may function analogously to the checkpoints in yeast which allow time for DNA repair prior to S and M phase transit, respectively [41, 42]. In support of a model in which TGF-β3 contributes to delaying cell cycle progression until DNA damage is repaired, we recently demonstrated that TGF-β3 can protect the intestinal crypt epithelium from radiation-induced toxicity *in vitro* and *in vivo* [43].

TGF-β growth arrest *per se* may not represent the only mechanism of chemoprotection, since other G_1 arrest mechanisms have also been exploited to promote the survival of cells treated with anticancer agents. A recent example is the p53 tumor suppressor [44–48], a component of a cell cycle checkpoint that is activated by DNA damage to promote either apoptosis or growth arrest, depending on the cell type and the expression of transforming oncogenes. In fibroblasts, activation of p53 by chemotherapy agents or radiation results in increased levels of the CDK inhibitor $p21^{Waf\text{-}1}$ [49], and a subsequent G_1 arrest [50]. In contrast,

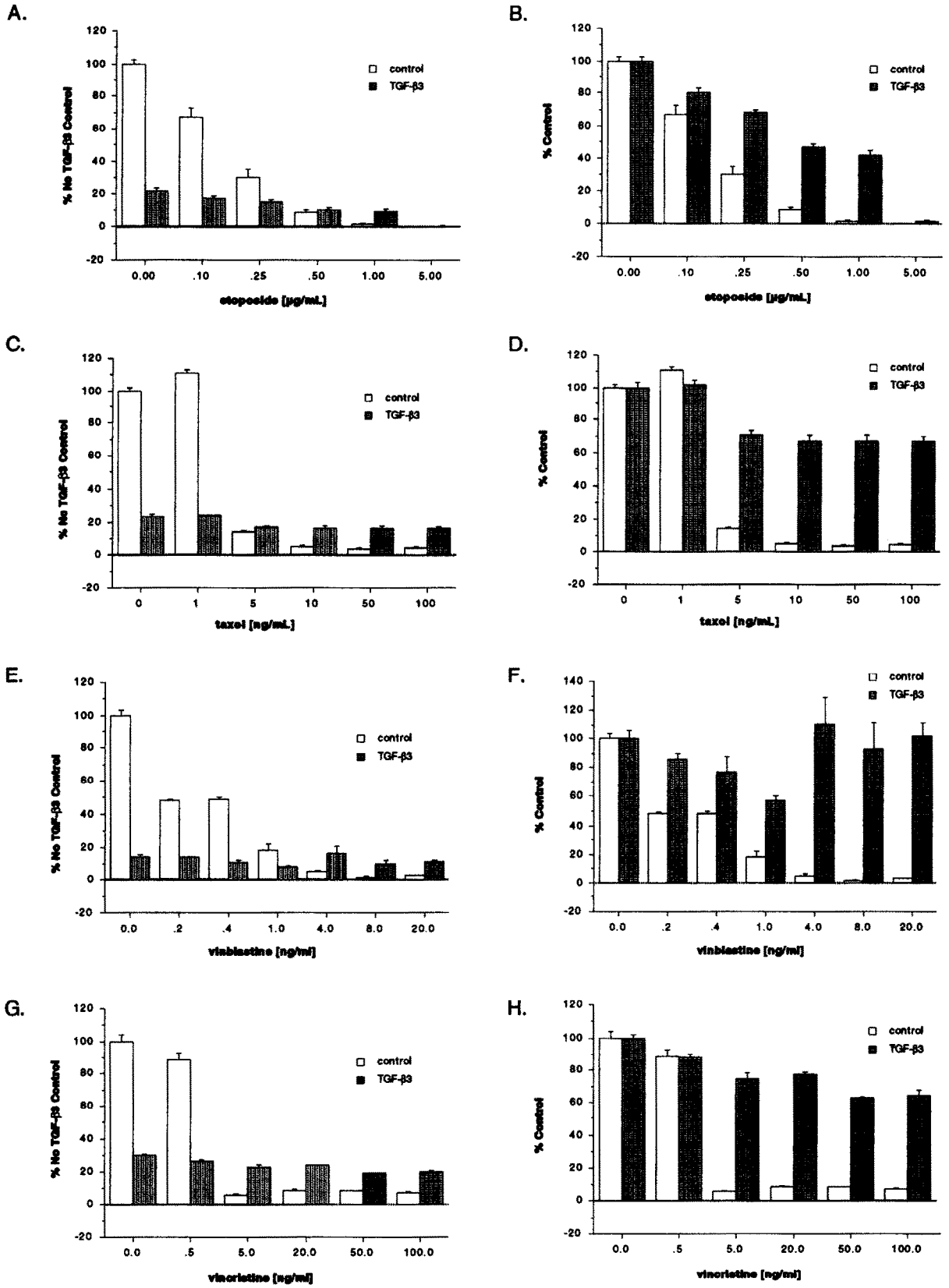

FIG. 5. TGF-β3 protection of CCL64 cells from the antimitotic agents etoposide, taxol, vinblastine, and vincristine. Drugs were added to cultures of cells, either in log-phase growth or after growth arrest with 15 pM TGF-β3. Colony counts were either tabulated as a percentage of the colonies formed from wells receiving neither TGF-β3 nor drug (A, C, E, and G), or the TGF-β3-treated and control colony counts were tabulated separately, with each of the no-drug (DMSO only) control counts normalized to 100% (B, D, F, and H). Each point is the mean of six replicates from one (vincristine), two (vinblastine), or three (etoposide and taxol) experiments, where error bars indicate the standard errors of the means.

TABLE 1. Modulation of cytotoxic IC_{50} by TGF-β3 *in vitro*

Cytotoxic agent	IC_{50} (μg/mL) No TGF-β3	IC_{50} (μg/mL) 15 pM TGF-β3
ara-C (N = 6, 1)	0.04	> 25
MTX (N = 6, 1)	< 0.05	> 50
5-FU (N = 6, 3)	0.5	1.0
Etoposide (N = 6, 3)	0.15	0.45
Taxol (N = 6, 3)	0.002	> 0.1
Vinblastine (N = 6, 2)	0.0002	> 0.02
Cisplatin (N = 6, 3)	0.3	0.3
Doxorubicin (N = 6, 3)	0.0011	0.0015

The half-maximal cytotoxic drug concentration (IC_{50}) required for growth inhibition of CCL64 epithelial cells, with or without TGF-β3 pretreatment. The number of replicate determinations from independent experiments are indicated in parentheses.

fibroblasts bearing either p53 mutations, no p53, or viral oncoproteins can continue to cycle immediately following DNA damage [51]. Such p53-deficient cells have been found to be more sensitive to ionizing radiation [45–47], paclitaxel [44], or other drugs [48] than their wild-type p53-expressing counterparts. Similar results have been obtained in our laboratory, using temperature-sensitive p53 mutations to induce growth arrest prior to drug exposure. Notably, p53 was similar to TGF-β3 in its profile of activity against different drugs: increasing survival after treatment with ara-C, taxol, vinblastine and etoposide, but not after cisplatin.**

Intracellular signals, mediated via binding of the TGF-βs to high-affinity cell surface receptors, appear to decrease the activity of the CDKs that operate specifically in G_1 [52–54]. While the precise mechanism(s) by which TGF-βs inhibit CDK activity appears to vary with the cell type, a general observation is that TGF-β signals lead to the increased expression or accessibility of one or more members of the CDK inhibitor family of heat-stable proteins. Observations that many tumors are deficient in the ability to arrest in response to anti-mitogenic signals that target various cell cycle checkpoints [22–25] favor the use of TGF-βs over metabolic inhibitors such as cycloheximide and 2-deoxy-D-glucose [55] as prospective chemoprotecting agents *in vivo*.

Prophylactic administration of TGF-β3 to the oral epi-

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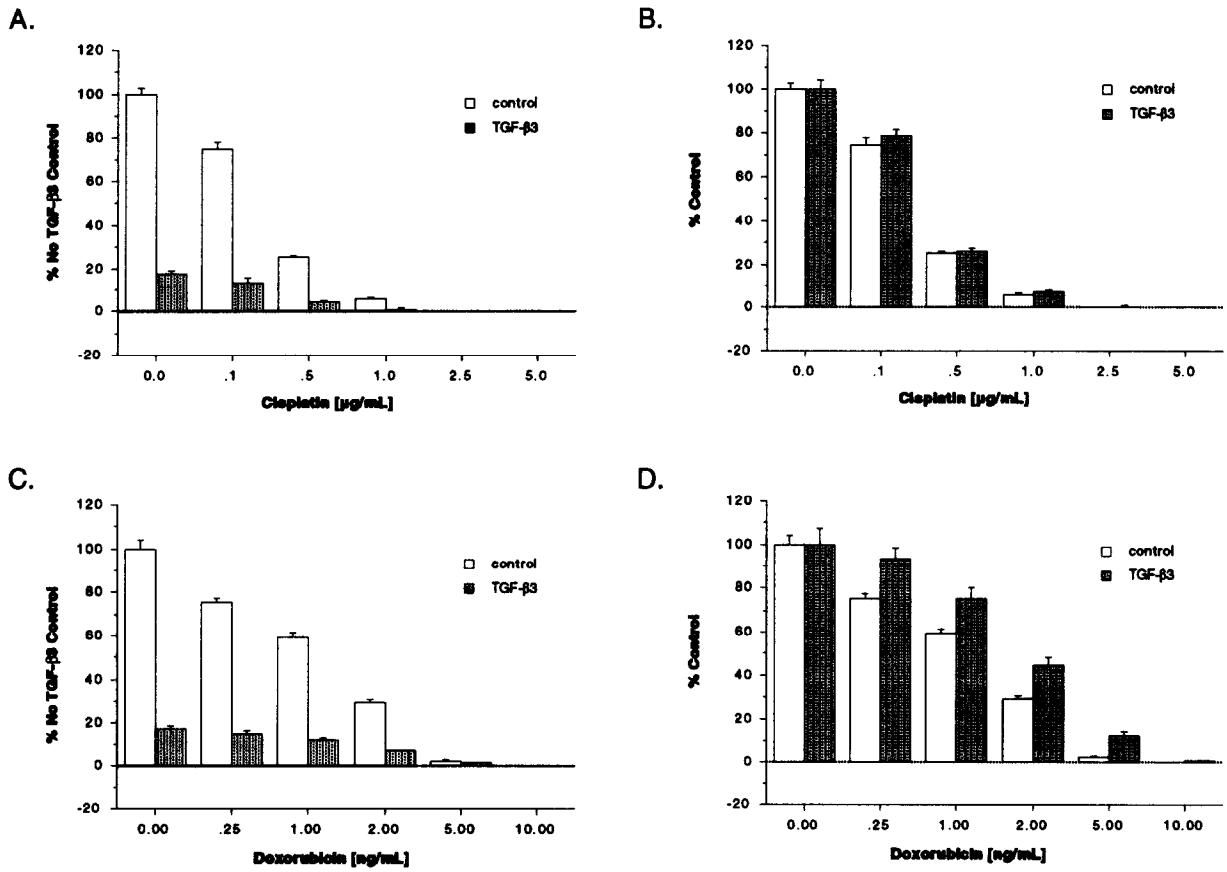

FIG. 6. Effects of TGF-β3 arrest on colony inhibition mediated by cisplatin and doxorubicin. Drugs in the concentrations shown were added to CCL64 cells in log-phase growth, or after growth arrest with TGF-β3. Colony counts were either tabulated as a percentage of the colonies formed from wells receiving neither TGF-β3 nor drug (A and C), or the TGF-β3-treated and control populations were treated separately, with each of the no-drug (DMSO only) control counts normalized to 100% (B and D). Each point is the mean of six replicates from three experiments each, where error bars indicate the standard errors of the means.

thelium of hamsters was shown previously to attenuate significantly ulcerative mucositis in response to 5-FU [34]. In CCL64 cells incubated with the same drug, however, TGF-β3 was only modestly chemoprotective. Similarly, TGF-β1 and TGF-β2 have been shown to protect mice from toxic doses of doxorubicin, an agent whose cytotoxicity was modestly, but significantly, modulated by TGF-β3 in the present study. One explanation for the disparity between the apparent magnitude of the *in vivo* and *in vitro* results may be that the clonogenic assay described here may be the more stringent of the two models: in the colony-forming assays, cells are plated at low density (where cell–cell contact is limited), whereas cell–cell contact in the oral epithelium is initially maintained in the animal model [34]. In addition, TGF-β3 pretreatment reduces the starting cell number for colony formation *in vitro*. In the animal model [34], the target cell population (oral epithelium) is proliferative, but absolute cell number is kept relatively constant due to a balance between cell division and cell death. In the animal system, short-term exposure did not reduce overall cellularity, and chemoprotection from 5-FU was readily observable. *In vivo*, other functions of the TGF-βs may also be relevant in promoting tissue chemoprotection and animal survival, for example, through a stimulation of neovascularization of the injured tissue [57, 58] and through the recruitment of fibroblasts [59] and monocytes [60] to sites of tissue damage. The available data suggest that TGF-βs should be examined further as a possible strategy to protect normal tissues from a broad range of chemotherapy protocols.

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