Differential Taxol-dependent Arrest of Transformed and Nontransformed Cells in the G1 Phase of the Cell Cycle, and Specific-related Mortality of Transformed Cells

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Abstract. Taxol (paclitaxel) induces a microtubule hyperassembled state, and effectively blocks cells in mitosis. Here we report that Taxol also induces a stable late-G1 block in nontransformed REF-52 and WI-38 mammalian fibroblast cells, but not in T antigen–transformed cells of the same parental lineage. G1 arrest is characterized by partially dephosphorylated pRb, and inactive cdk2 kinase. Nontransformed cells recover normally from Taxol arrest. In contrast, T antigen transformed cells continue inappropriately past both G1 and G2-M in the presence of Taxol, and undergo a rapid death upon release. These results demonstrate a microtubule sensitive step in G1 regulation of nontransformed fibroblast cells. Also, Taxol selectively induces death of transformed cells, possibly because they slip the Taxol-dependent G1 arrest, as well as G2/M arrest, which are both specific to nontransformed cells.

MICROTUBULES play distinct roles at different stages of the cell cycle. During interphase, they establish cell polarity, are involved in the organization of the cytoplasm, and play a vital role in vesicle transport. During mitosis, microtubules form into a mitotic spindle that is centrally important both in chromosome congression to the metaphase plate and in chromatid separation during anaphase.

In mitosis, the role of microtubules in regulating mitotic exit extends beyond their mechanical function in chromosome movement. Microtubules also have an intrinsic regulatory function in mitotic exit, perhaps serving as the site of degradation of cyclin B and for inactivation of $p34^{cdc2}$ kinase (Kubiak et al., 1993; Andreassen and Margolis, 1994). We have recently found that there is a strong correlation between the assembly state of microtubules and the metabolic ability of the cell to inactivate $p34^{cdc2}$ and induce mitotic exit (Andreassen and Margolis, 1994). Nocodazole inhibits microtubule assembly in a concentration-dependent manner (Hoebeke et al., 1976; Jordan et al., 1992), and concentrations of nocodazole that block microtubule assembly also inhibit the activation of the mitotic exit machinery. In contrast to nocodazole, Taxol (paclitaxel) induces hyperassembly of microtubules (Schiff et al., 1979; Jordan et al., 1993). BHK cells blocked with Taxol in mitosis undergo inappropriate mitotic exit, either through the checkpoint override activity of the protein kinase inhibitor, 2-aminopurine, or as a result of natural "mitotic slippage" (Andreassen and Margolis, 1994).

The demonstration that microtubules have an intrinsic regulatory function in the control of mitotic exit has raised the intriguing prospect that such microtubule-dependent control could extend to other stages of the cell cycle. Here, we report that microtubules are also involved in regulating the transition from G1 to S phase in nontransformed fibroblast cells. We find that, in addition to its capacity to induce mitotic arrest, Taxol causes normal mammalian cells to arrest indefinitely late in the G1 phase of the cell cycle. Furthermore, we find that Taxol-dependent G1 arrest occurs only in nontransformed cells, and that SV40 large T antigen transformed cells of the same parental lineage do not arrest in G1 in response to Taxol. Since the arrest we observe is unique to nontransformed cells, our results suggest the presence of a microtubule sensitive step in the G1 regulatory machinery of nontransformed cells.

The presence of both late G1 and mitotic controls that are sensitive to the state of microtubule assembly suggests that microtubules may be of general importance to the regulation of cell cycle progression. We have observed that Taxol-treated transformed cells fail to arrest stably both in G1 and in G2/M, and exhibit inappropriate mitotic exit through slippage, while the parental line arrests stably at both points. The absence of sustained mitotic or G1 arrest in the presence of Taxol correlates with our finding that the drug has a remarkable ability to selectively induce death in transformed fibroblast cells. Thus, we find in accord with a recent report (Wahl et al., 1996), that transformed cells treated with Taxol rapidly and quantitatively die in protocols where nontransformed cells of the same

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lineage survive Taxol arrest and proliferate upon release. While Wahl et al. (1996) observed G1 arrest in nontransformed cells, they interpreted the arrest as a late consequence of DNA damage as Taxol-treated cells passed through mitosis. For the first time, we demonstrate here that Taxol causes nontransformed cells to arrest in G1 as a primary event, and not as a result of previous DNA damage.

These results show that Taxol is selectively lethal to T antigen–transformed tumor cells, possibly by subversion of drug-induced mitotic and G1 arrest. Taxol is a promising drug in the treatment of breast and ovarian tumors (Rowinsky and Donehower, 1995). These results may offer an explanation of the apparent efficacy of Taxol in tumor therapy. Our results also suggest the possibility of an assay to screen for other drugs that might exploit the propensity of certain tumor cells to evade the microtubule sensitive G1 and G2/M arrest points.

Materials and Methods

Cell Culture

REF-52 (rat primary fibroblasts) and SV40 large T antigen–transformed progeny cells (TAG)[1] (Perry et al., 1992) were a generous gift of G.R. Stark (Cleveland Clinic Foundation, Cleveland, OH). Human primary fibroblasts WI-38 and SV40-transformed progeny cells (VA-13) were obtained from Amer. Type Culture Collection (Rockville, MD). REF-52 and WI-38 primary cell lines were used at less than 30 and 10 passages, respectively. Cell doubling times at mid-log phase for the various cell lines used are REF-52, 24 h; TAG, 18 h; WI-38, 40 h; and VA-13, 24 h.

All cells were cultured as monolayers in DMEM (GIBCO BRL, Paisley, UK) supplemented with 10% FBS (Biological Industries, Israel). Cells were maintained in a humid incubator at 37°C in a 5% CO_2 environment.

Synchronization

Contact inhibited cells were prepared by maintaining populations at confluency for 48 h. Cells were released from contact inhibition by trypsinization and replating as subconfluent cultures. Taxol at 5 μg/ml, nocodazole at 0.5 μg/ml, or hydroxyurea at 2 mM were applied 2 h before release from contact inhibition. Taxol is a trademark name (Bristol-Myers) for paclitaxel.

Taxol, nocodazole, and hydroxyurea were also applied to cycling cells at 5 μg/ml, 0.5 μg/ml, and 2 mM, respectively. Taxol, nocodazole, and hydroxyurea were obtained from Sigma Chem. Co (St. Louis, MO). Taxol and nocodazole were prepared as stock solutions in DMSO at 5 mg/ml and 1 mg/ml, respectively. Hydroxyurea was prepared as a 200-mM stock solution in DMEM containing 10% FBS.

Immunofluorescence Microscopy

Cells were grown on poly-lysine–coated glass coverslips. For immunolocalization of microtubules, cells were fixed 20 min at 37°C with 2% paraformaldehyde in PBS, washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 3 min, and washed with PBS. Anti–β-tubulin ascites antibodies (Sigma Chemical Co., TUB 2.1) were diluted 400-fold. Incubation with primary and secondary antibodies, washes, and counterstaining with propidium iodide were performed as previously described (Andreassen et al., 1991).

For immunolocalization of PCNA, cells were fixed with 1% formaldehyde at RT for 2 min, and were then postfixed with methanol at −20°C for 10 min, and treated with 0.5% NP-40 for 2 min (Ogata et al., 1987). Cells were washed with PBS, and then incubated with primary antibodies (Coulter, Miami, FL) diluted 50-fold. Cells were then washed, incubated with secondary antibodies, and counterstained with propidium iodide. For all immunofluorescence, primary antibodies were detected with FITC-conjugated goat anti–mouse IgG secondary antibodies at 2.5 μg/ml.

For microscopy, coverslips were mounted as previously described (Andreassen et al., 1991) and observed using an MRC-600 Laser Scanning confocal apparatus (Bio-Rad Microscience Division, Herts, England). Photographs were taken on TMAX-100 film with a Polaroid CI-3000 film recorder.

Flow Cytometric Analysis

Cells were collected by trypsinization, pooled with nonattached cells, resuspended in PBS, and fixed by the addition of methanol to 90% at −20°C. After 10 min fixation, cells were pelleted, resuspended, and stored in PBS with 0.04% sodium azide. Cells were prepared for flow cytometry by washing with PBS and resuspending in 4 mM sodium citrate containing 30 U/ml RNase A, 0.1% Triton X-100, and 50 μg/ml propidium iodide and incubated for 10 min at 37°C. Sodium chloride was then added at 138 mM and samples were kept on ice until analyzed.

Data were collected using a FACScan (Becton Dickinson & Co., San Jose, CA) and results were analyzed with LYSYS II software. For each sample, 5,000 events were collected and aggregated cells were gated out.

Immunoblotting

To prepare immunoblots, 20 μg of whole cell lysates were resolved on polyacrylamide gels. Semi-dry transfer of proteins to nitrocellulose for detection of all antigens (except pRb, which was transferred to Immobilon) and blocking with 5% nonfat milk in TNT buffer (25 mM Tris, pH 7.5, 150 mM sodium chloride, and 0.05% Tween 20) was as previously described (Andreassen and Margolis, 1994). Blots were washed twice with TNT and incubated 3 h with primary antibodies in TNT. Primary antibodies used were anti–human Rb (Pharmingen, San Diego, CA) and anti-p27 (Transduction Laboratories, Lexington, KY) diluted 2,000-fold; anti-$p21^{WAF1/CIP1}$ (C19) and anti-cdk4 from Santa Cruz Biotechnologies (Santa Cruz, CA) diluted 500-fold; and anti-cdk2 antiserum (Brénot-Bosc et al., 1995), the kind gift of R. Fotedar (Inst. de Biologie Structurale, Grenoble, France), diluted 3,000-fold. Washes, incubation with HRP-conjugated goat anti–mouse and anti–rabbit IgG secondary antibodies, and revelation by enhanced chemiluminescence (Amersham Corp, Buckinghamshire, UK) was as previously described (Andreassen and Margolis, 1994).

Cdk2 Kinase Assay

REF-52 cells at contact inhibition, or from populations released from contact inhibition into either 5 μg/ml Taxol or 2 mM HU for 20 h were collected by trypsinization, washed with cold PBS, and cell lysates were prepared in 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EGTA, 0.1% NP-40 containing 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 60 mM β-glycerophosphate, 50 mM NaF, and 0.5 mM sodium vanadate as previously described (Andreassen and Margolis, 1994).

40 μg of each extract was incubated with 25 μl of protein A–Sepharose 4B beads for 30 min at 4°C to preclear proteins that bind nonspecifically to the beads. 4.0 μl of anti-cdk2 antiserum (Brénot-Bosc et al., 1995) was added to the extract for 1 h at 4°C. Then, 50 μl of protein A–Sepharose beads was added for 1 h at 4°C. The resulting immune complex was washed three times with lysis buffer and one time in kinase buffer. The pellet was resuspended in 50 μl of kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA) containing 1 μg histone H1, 30 μM ATP, and 5 μCi of [γ-^{32}P]ATP. The H1 kinase assay was carried out for 30 min at 37°C and was terminated by the addition of sample buffer. Samples were then resolved by SDS-PAGE using 12% polyacrylamide gels (19:1 ratio of acrylamide to *bis*-acrylamide) (Andreassen and Margolis, 1994). Autoradiographs were prepared by exposure to Hyperfilm-MP (Amersham Corp.).

Cdk4 Kinase Assay

REF-52 cells at contact inhibition, or from populations released from contact inhibition into either 5 μg/ml Taxol or 2 mM HU for 20 h were collected by trypsinization and washed with 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, and 1 mM dithiothreitol. Cells were then lysed in the above buffer supplemented with 10% glycerol, 0.1% Tween 20, 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium vanadate by sonication two times for 10 s according to the protocol of Matsushime et al.

1. *Abbreviations used in this paper*: 2-AP, 2-aminopurine; HU, hydroxyurea; TAG, T-antigen transformed REF-52 cell; TUNEL, transferase-mediated dUTP-biotin nick end labeling.

(1994) and left on ice for 30 min. Lysates were clarified by centrifugation 5 min at 12,000 *g*.

40 μg of each extract was incubated with 10 μl of protein G–Agarose beads for 60 min at 4°C to preclear proteins that bind nonspecifically to the beads. 10 μl of anti-cdk4 antibody (goat polyclonal IgG C-22, Santa Cruz Biotechnologies) was added to the extract for 1 h at 4°C. The resulting immune complex was washed three times with lysis buffer and one time in kinase buffer. The pellet was resuspended in 50 μl of kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium vanadate) containing 0.5 μg of pRb substrate (carboxy terminal peptide, Santa Cruz Biotechnologies), 20 μM ATP and 10 μCi [γ^{32}P]ATP. The kinase reaction was conducted for 30 min at 30°C. Samples were resolved by SDS-PAGE using 12% polyacrylamide gels (30:0.8 ratio of acrylamide to *bis*-acrylamide).

Analysis of Cell Death and Apoptosis

Cells were grown as monolayers and treated as described in the text. At timepoints, cells from individual plates initially seeded at equal cell density were collected by trypsinization and resuspended in trypan blue (Sigma Chem. Co, St. Louis, MO) diluted to 0.1% in PBS for 5 min at RT. Viable cells, distinguished by the exclusion of trypan blue, were counted using a Thoma counting chamber (Germany). All data points consisted of three counts of at least 100 living cells. The standard deviation was <5% of the ordinate value for all data points.

Apoptotic cells growing on coverslips were detected microscopically using terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL). After appropriate drug treatment, TAG cells were fixed with 1% formaldehyde in PBS for 15 min at ambient temperature. Cells were permeabilized 2.5 min with 0.5% Triton X-100 in PBS, and washed two times 5 min each with 25 mM Tris-HCl, pH 6.6, containing 200 mM potassium cacodylate and 2.5 mM cobalt chloride. Cells were then incubated with the above buffer containing 0.25 mg/ml bovine serum albumin, 100 U/ml terminal deoxynucleotide transferase, and 10 μM biotin 16-dUTP for 45 min at 37°C. Cells were then washed with PBS, and then incubated with 60 mM trisodium citrate and 600 mM sodium chloride containing 0.1% Triton X-100, 5% nonfat milk and 2.5 μg/ml FITC-avidin for 1 h at 37°C. Samples were then washed with PBS and mounted. For quantification of apoptosis, three counts of 200 cells each were made for each sample.

Results

Taxol Arrests REF-52 and WI-38 Cells with Both 2N and 4N DNA Content, but Arrests Their Transformed Variants with 4N Content Only

After continuous exposure of random cycling rat embryo fibroblasts (REF-52) primary nontransformed cells to 5 μM Taxol, flow cytometric analysis shows the presence of two distinct populations. After 20 h of treatment with Taxol, the population displays two sharp peaks, representing 2N and 4N DNA content (Fig. 1 *A*). In these cells, the 2N and 4N peaks appear stable, yielding essentially the same population distribution after 40 h of exposure to Taxol (33% 2N), compared to 20 h of exposure (36% 2N) (Fig. 1 *A*). After 20 h (cell doubling time in the absence of drugs is 24 h) of exposure to nocodazole, REF-52 cells have almost entirely transited through the cell cycle, and are nearly all arrested in a 4N state that remains stable at 40 h (Fig. 1 *A*). The possibility can therefore be excluded that a portion of the population remains 2N because it is not in the cell cycle during the time course of exposure to Taxol.

Taxol exposure arrests REF-52 cells at a point before the onset of S phase. If, instead, REF-52 cells are blocked with hydroxyurea (HU) for 15 h, and then released from the HU block into 5 μM Taxol, the population is entirely 4N after a total elapsed time of 25 h (see Fig. 3). We conclude that the point of Taxol blockage is most likely in the G1 phase of the cell cycle, as it neither coincides with nor comes after the point of blockage with HU, which occurs early in S phase (Walters et al., 1976).

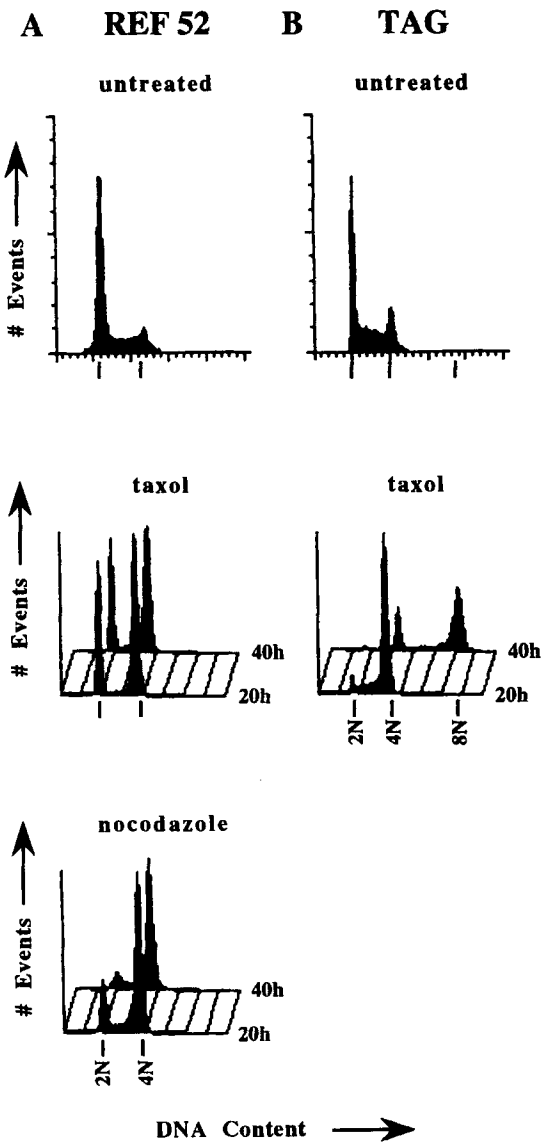

Figure 1. Taxol arrests random cycling REF-52 cells but not TAG cells as a 2N population. Random cycling ("untreated") REF-52 and TAG cells were grown to ~30% confluency, and then treated with 5 μM Taxol for either 20 or 40 h. After 20 or 40 h of treatment with Taxol, REF-52 cells are stably arrested as both 2N and 4N populations. In contrast, TAG cells do not exhibit either stable 2N or 4N arrest. At 20 h they are 4N, but by 40 h they have largely evaded mitotic arrest and progressed through the next cell cycle and have 8N DNA content. In contrast to the effect of Taxol, 1.0 μg/ml nocodazole yields a stable 4N block in REF-52 cells. Data were collected by flow cytometry.

In sharp contrast to the above results, REF-52 cells that have been transformed by introduction of SV40 large T antigen, designated TAG cells, exhibit no Taxol-dependent arrest in a 2N state (Fig. 1 *B*). Instead, after a period of exposure to Taxol for 20 h, randomly cycling TAG cells exhibit a 4N peak. After 40 h in Taxol, a substantial fraction of the population has undergone mitotic slippage and

has completely transited through another cell cycle, accumulating as 8N cells (Fig. 1 *B*).

In summary, nontransformed REF-52 cells arrest in their cell cycle as both 2N and 4N cells in response to Taxol. While nocodazole or Taxol will arrest the cells in a 4N state, 2N arrest arises uniquely from Taxol, and is not duplicated with nocodazole. Large T antigen transformation of REF-52 cells completely abrogates the 2N arrest point, and may introduce an element of mitotic slippage.

We next addressed whether the 2N arrest in response to Taxol arises in another nontransformed cell line. Random cycling primary human lung cells (WI-38) were exposed to 5 μM Taxol, and after 40 h of exposure (cell doubling time is 40 h) they also exhibited sharp peaks at 2N and 4N DNA content (Fig. 2 *A*). Again, in common with REF-52 cells, the point of arrest in these cells is at late G1. WI-38 cells released from contact inhibition into HU for 20 h arrest at S phase onset. When released from HU into 5 μM Taxol for an additional 20 h, they then transit from early S phase to mitosis, yielding a strong 4N peak (Fig. 2 *A*).

In contrast, SV40-transformed WI-38 cells, designated VA-13, arrested almost exclusively as a 4N population after 20 h of exposure to Taxol (cell doubling time is 24 h) (Fig. 2 *B*). In this case, there was no difference in the profile of the arrested population, regardless of whether they were exposed continuously to Taxol for 20 h, or were first arrested with HU for 10 h then released into Taxol for an additional 10 h (Fig. 2 *B*). Thus, nontransformed human lung cells, but not their transformed counterparts, have the capacity to arrest with 2N DNA content in the presence of Taxol.

Release of REF-52 Cells from Contact Inhibition into Taxol Yields a Population Blocked with 2N DNA Content

The arrest of REF-52 cells in a 2N state can be enhanced by releasing contact inhibited cells into 5 μM Taxol. Contact inhibition induces arrest of various primary cells in late G1 (Polyak et al., 1994*a*; Deffie et al., 1995). Cells in a contact inhibited state were treated with Taxol for 2 h before release, and then trypsinized and replated in the presence of 5 μM Taxol. After 25 h in Taxol, the population, when analyzed by flow cytometry, was found to be almost entirely 2N in DNA content (Fig. 3). This state persisted through 35 h of continuous exposure to Taxol (Fig. 3). In contrast, cells exposed first to HU for 15 h during release from contact inhibition, and then released from HU into Taxol for an additional 10 h were almost entirely 4N (Fig. 3), demonstrating that 35 h was sufficient time for the entire population to transit the cell cycle from a contact inhibited state to Taxol arrest at G2/M.

Interestingly, cells exposed to 0.5 μg/ml nocodazole upon release from contact inhibition consistently approached a 4N state later than released cells that were treated with HU, and then with Taxol (Fig. 3). After 25 h of exposure to nocodazole, the population shown was determined to be 21% G1, 28% S phase, and 48% G2/M, in contrast to cells treated for 15 h in HU, and then 10 h in Taxol, which were 82% G2/M. By 35 h, 72% of the cells released from contact inhibition into nocodazole had reached G2/M. We conclude that transit through the early

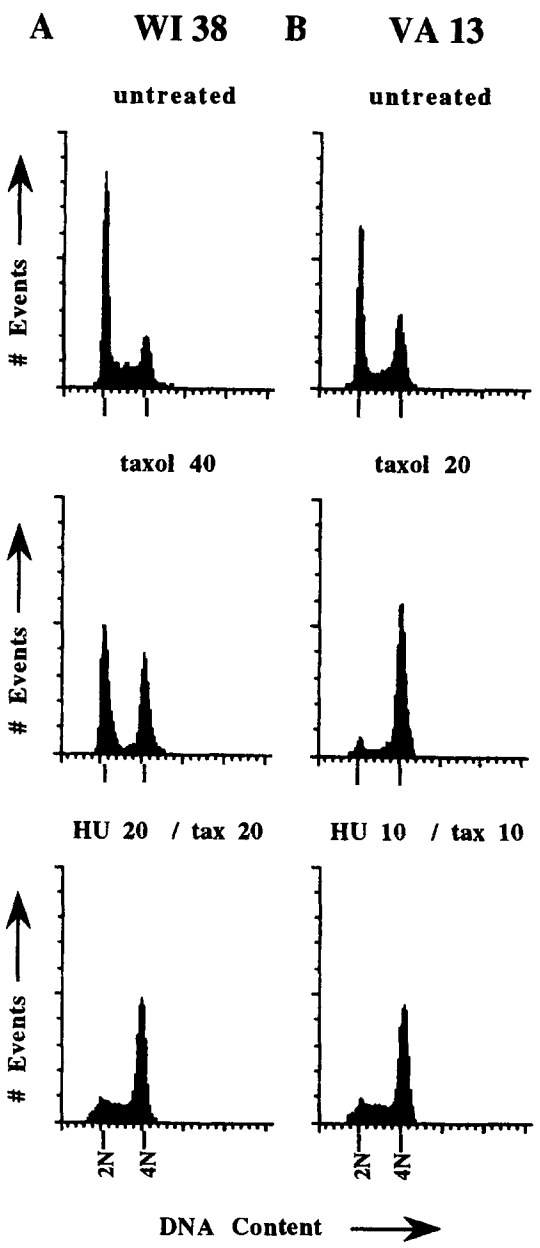

Figure 2. Taxol arrests random cycling WI-38 cells, but not VA-13 cells, with 2N DNA content. Random cycling ("untreated") WI-38 (*A*; *top image*) and VA-13 (*B*; *top image*) cells were grown to ~30% confluency, and then treated with 5 μM Taxol for 20 (VA-13) or for 40 (WI-38) h. At 40 h the WI-38 cells are 40% 2N, while the VA-13 cells are nearly all 4N by 20 h. WI-38 cells (*A*; *bottom image*), first released from contact inhibition into HU for 20 h to induce arrest in early S phase, then released into Taxol for an additional 20 h, arrest as a largely 4N population. Data were collected by flow cytometry.

cell cycle is also sensitive to nocodazole-dependent disassembly of microtubules, yielding a lag in cell cycle progression as opposed to the absolute G1 block imposed by Taxol.

The microtubule network of cells released from contact inhibition into various drugs was examined by immunocytochemistry, revealing that 20 h of exposure to Taxol produced extensive bundles of cytoplasmic microtubules (Fig. 4 *A*), while exposure to nocodazole totally disassembled

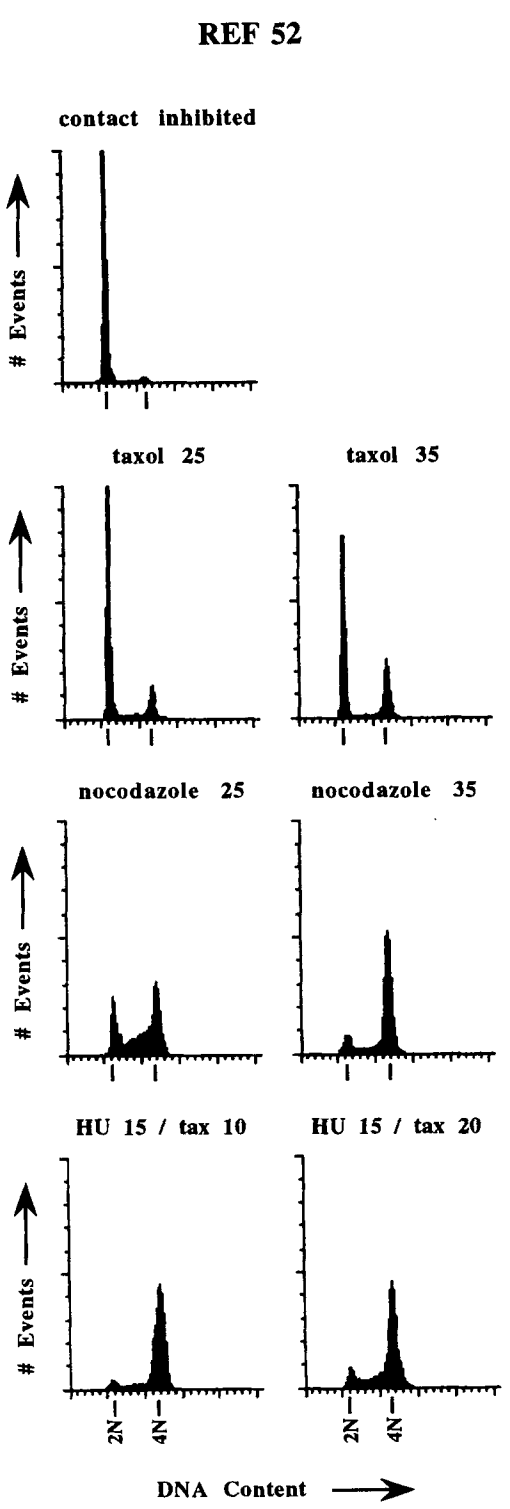

Figure 3. REF-52 cells, released from contact inhibition into Taxol, arrest predominantly with 2N DNA content. REF-52 cells were grown to confluency, then left as a contact inhibited population for 48 h before release. Cells were released from contact inhibition by replating at 30% confluency. For Taxol treatment, cells were preincubated with 5 μM Taxol for 2 h before release, then maintained in Taxol for the times indicated. After 25 h in Taxol, 76% of the population is 2N; after 35 h, 67% is 2N. Other cells were similarly treated with 0.5 μg/ml nocodazole, or with hydroxyurea (HU) for 15 h, then with Taxol for either 10 or 20 h, as indicated. In these cases, the cell population progresses to 4N DNA content. Data were collected by flow cytometry. In all cases, numbers indicate hours of drug treatment after release from contact inhibition.

the interphase array of microtubules over the same time course (data not shown). By comparison, HU-blocked cells showed a normal interphase array of microtubules (Fig. 4 *A*). Thus, the G1 arrest induced by Taxol correlates with rearrangement and stabilization of the microtubule array.

Analysis of the G1 Arrest Status of Taxol-treated REF-52 Cells

Release of REF-52 cells from contact inhibition into Taxol yields a uniform population of 2N cells, permitting biochemical analysis of their arrested state. PCNA, a subunit of the DNA polymerase complex, is present in nuclei only during S phase (Bravo and MacDonald-Bravo, 1985). Thus, following release of REF-52 cells from contact inhibition into HU for 20 h, their nuclei are uniformly positive for PCNA (Fig. 4 *A*), indicating that the population has entered early S phase by this time. In contrast, cells released from contact inhibition into Taxol for the same period of time have 2N DNA content (Fig. 3), but uniformly contain no intranuclear PCNA stain (Fig. 4 *A*). The lack of PCNA stain in nuclei of such cells persists indefinitely in the continuous presence of Taxol (data not shown). Thus, cells released from contact inhibition into Taxol remain stably arrested in late G1. This conclusion comes from the lack of PCNA stain in the nuclei of arrested cells, and from evidence that the point of Taxol arrest precedes the HU block point.

We have analyzed the Taxol-arrested cell population for the presence of distinctive late G1 markers. $p21^{CIP1/WAF1}$ and $p27^{Kip1}$ are specific inhibitors of the cdk kinases required for the G1/S phase transition (Harper et al., 1993; Polyak et al., 1994*b*; Toyoshima and Hunter, 1994). $p27^{Kip1}$ is upregulated in quiescent cells and is abruptly degraded at the G1-S transition (Nourse et al., 1994; Pagano et al., 1995). Western blot analysis demonstrates that $p27^{Kip1}$ is prominent in contact inhibited REF-52 cells, but is at minimal levels in cells blocked at S phase with HU (Fig. 4 *B*). REF-52 cells released from contact inhibition into Taxol contain $p27^{Kip1}$ levels comparable to those of contact inhibited cells. $p21^{CIP1/WAF1}$ is present in contact inhibited REF-52 cells and remains present at diminished levels in Taxol-treated cells. This result is in sharp contrast to Wahl et al. (1996), who found induction of $p21^{CIP1/WAF1}$ in cells treated with Taxol following synchronization at S phase, and interpreted the increase as due to a DNA damage response. Our results show that Taxol-dependent G1 arrest is not characteristic of that following DNA damage and that Taxol arrest does not appear to require $p21^{CIP1/WAF1}$ induction.

pRb, a protein involved in regulation of the G1-S transition, undergoes inactivation by CDK-dependent phosphorylation at the time of transition past the G1 restriction point (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989). Contact inhibited cells contain underphosphorylated pRb, while a slower migrating phosphorylated form is evident in HU-blocked cells (Fig. 4 *B*). In contrast, in cells released from contact inhibition into Taxol, pRB

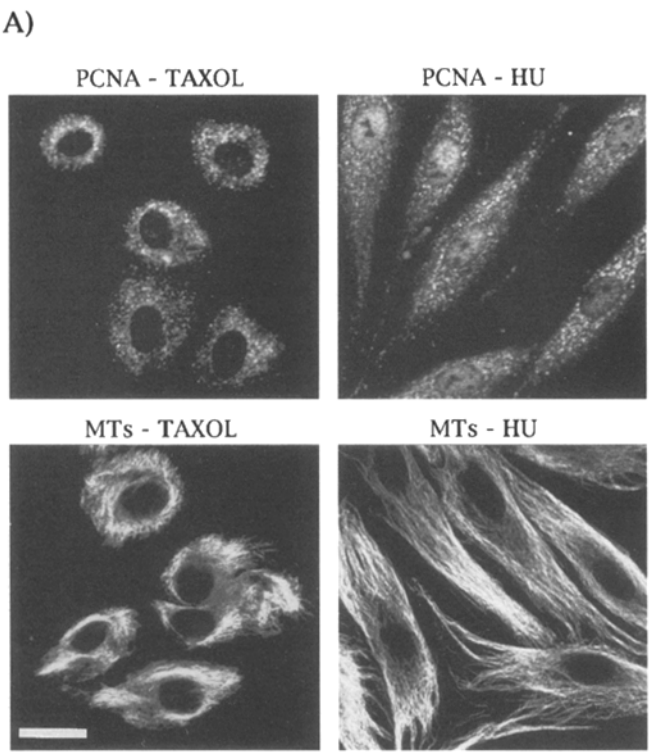

Figure 4. REF-52 cells released from contact inhibition into Taxol are arrested in late G1. (*A*) PCNA is excluded from nuclei of Taxol-arrested REF-52 cells (*above*, *left*). REF-52 cells were released from contact inhibition into either 5 μM Taxol or 2 mM hydroxyurea (HU). After 25 h of release, cells were fixed and prepared for immunofluorescence analysis. PCNA serves as an intranuclear S phase marker. HU-blocked cells have PCNA in their nuclei, whereas Taxol-blocked cells do not. Anti-tubulin antibody reveals a characteristic interphase array of microtubule (MTs) bundles in Taxol-treated cells (*below*, *left*), compared to the normal microtubule array present in HU-blocked cells (*below*, *right*). Bar, 20 μm. (*B*) Taxol arrested 2N REF-52 cells have markers characteristic of late G1. Contact-inhibited cells, or cells released from contact inhibition for 25 h into either Taxol or hydroxyurea (HU) were harvested. Then, to determine expression levels of various cell cycle proteins, samples were subjected to SDS-PAGE, and then exposed to the appropriate antibodies in Western blotting procedures. The kinase activities of cdk2 and cdk4 were determined following immunoprecipitation of the enzymes, using histone H1 or a pRb peptide as substrates, respectively. Autoradiographs of ^{32}P incorporation are shown.

maintains a stable state with approximately half of the pRb population phosphorylated. The partial phosphorylation status of pRb in cells released from contact inhibition into Taxol has been repeatedly observed in independently prepared extracts.

We have also analyzed the activity of both cdk2 and cdk4 protein kinases. Cdk4 is normally active during mid-to-late G1 (Matsushime et al., 1994; Sherr, 1994), whereas cdk2 is activated at the point of commitment to S phase, and is required for S phase entry (Fang and Newport, 1991; Pagano et al., 1993; Tsai et al., 1993). Both enzymes are present in cells at constant levels under all treatment conditions except for and increase in cdk2 in HU (Fig. 4 *B*). Cdk4 shows activity in contact inhibited cells and remains approximately as active in Taxol-arrested cells (Fig. 4 *B*). The kinase activity of cdk4 is, however, diminished in HU-treated cells. Our analysis of immune-precipitated cdk2 kinase activity shows that it is inactive in REF-52 cells during contact inhibition, but is active in extracts prepared from cells blocked in S phase with HU (Fig. 4 *B*). Cells released from contact inhibition into Taxol contain inactive cdk2 kinase (Fig. 4 *B*), consistent with all other markers demonstrating an indefinite G1 arrest in the presence of Taxol.

Correlation between Failure of G1 Arrest in Taxol and Drug Toxicity in Transformed Cells

There is a dramatic difference between transformed and nontransformed cells of the same parental lineage with respect to induction of cell death following exposure to Taxol. REF-52 cells arrest in the presence of Taxol and resume normal proliferation upon release. In striking contrast, TAG cells given the same drug regimen die following release.

TAG cells die within 6 d of release following different times of exposure to Taxol (Fig. 5 *A*). After an exposure of as little as 10 h (cell doubling time is 16 h), the majority of random cycling TAG cells ultimately die, although there is a brief period during which a small amount of proliferation occurs. After 20 or 40 h exposure to Taxol, cell death commences immediately and is complete by 6 d (Fig. 5 *A*). When REF-52 cells are treated identically, they arrest in the cell cycle, then recommence proliferating upon release from the drug, ultimately reaching confluency (Fig. 5 *B*). The capacity to resume proliferation is independent of the time of drug treatment, between 20 and 40 h, and also independent of whether the cells were randomly cycling or released from contact inhibition before drug exposure (data not shown).

The period of the cell cycle in which TAG cells are exposed to Taxol is important in their capacity to survive. After exposure to Taxol for 10 h, randomly cycling cells gave a brief proliferative response, and then proceeded to die (Fig. 5, *A* and *C*). In contrast, if the cells are first collected in early S phase by a 10-h HU block following contact inhibition, then released into Taxol for 10 h, they proceed to mitosis by the time Taxol is removed. Such cells

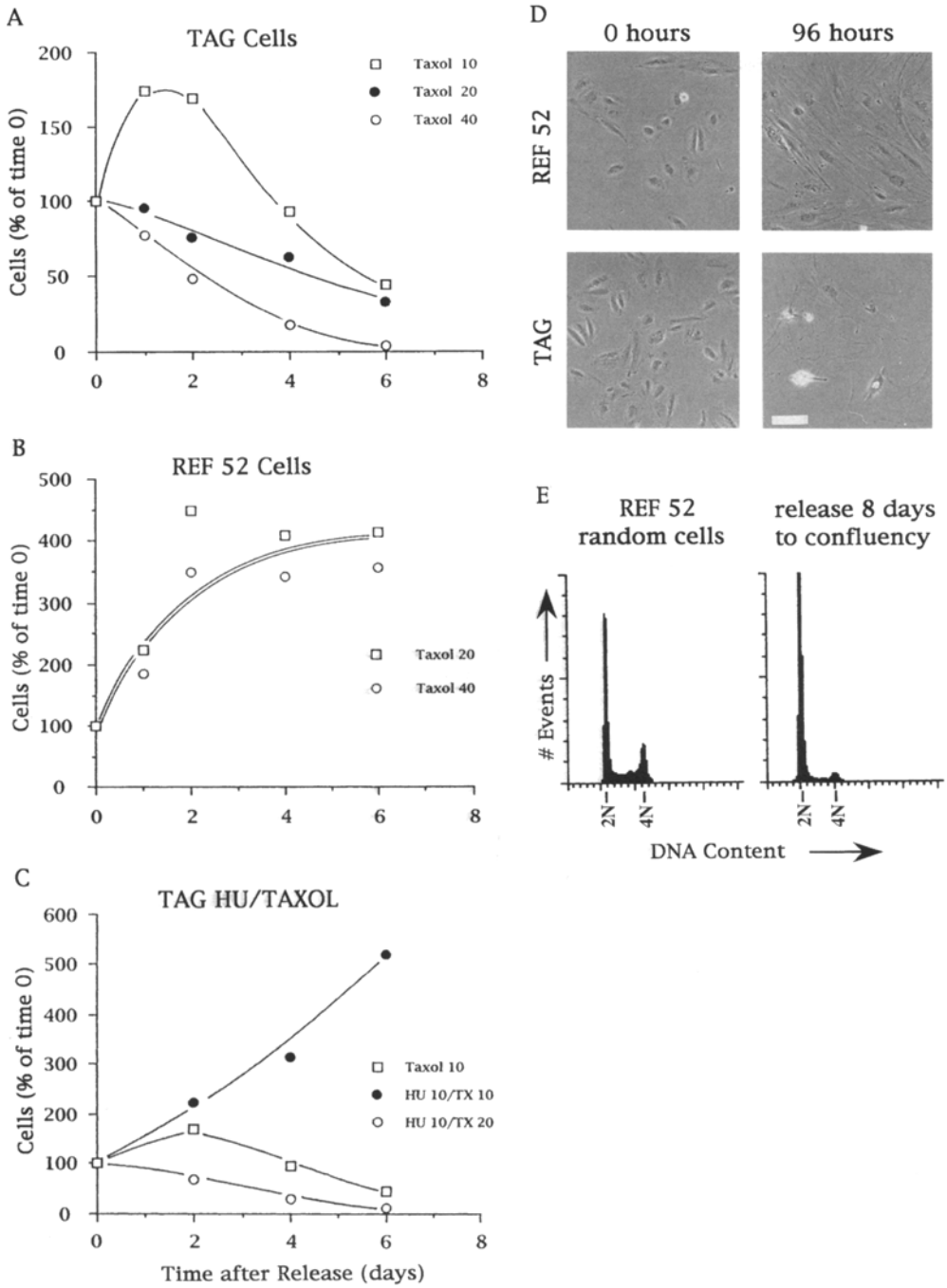

Figure 5. Relative survival of REF 52 and TAG cells following Taxol arrest. (*A*) Randomly cycling TAG cells were treated with 5 μM Taxol for 10, 20, or 40 h, as indicated, then released. At the indicated time points, cell counts were taken. (*B*) Randomly cycling REF 52 cells were treated with 5 μM Taxol for 20 or 40 h, as indicated, then released. At the indicated time points, cell counts were taken. The plateau at later times indicates approach to confluency. (*C*) Randomly cycling TAG cells were treated either with 5 μM Taxol for 10 h, or with hydroxyurea (HU) for 10 h, then with 5 μM Taxol for either 10 or 20 h, as indicated, and then released. At the indicated time points, cell counts were taken. (*D*) Phase contrast images of random fields of REF 52 and TAG cells taken at time 0 and at 96 h after release from 20 h of treatment with 5 μM Taxol. All pictures were taken at the same magnification. (*E*) Flow cytometric analysis of DNA content of REF 52 cells, comparing a randomly cycling population with cells treated with Taxol for 20 h, and then released. The sample was collected at contact inhibition, 8 d after release from Taxol. Bar: (*D*) 60 μm.

survive the drug treatment and continue to proliferate upon release (Fig. 5 *C*). If however cells are released from HU block into Taxol for 20 h, a period of time sufficient to allow cells to slip from mitotic arrest into the next cell cycle, the cells ultimately die after release from Taxol (Fig. 5 *C*). The contrast in survival demonstrates that the period of lethality in response to Taxol lies between late mitosis and entry into S phase.

The relative survival of cells is evident visually in phase contrast images of random fields of cells. After release from 20 h of Taxol treatment, nontransformed REF-52 cells recommence proliferation, while transformed cells rapidly die. The difference in cell density in the populations after 48 h of release from Taxol is striking (Fig. 5 *D*).

REF-52 cells were analyzed by flow cytometry 8 d after release from 20 h treatment with Taxol, after reaching a state of contact inhibition. These cells have euploid DNA content indistinguishable from that of REF-52 cells that were never Taxol treated (Fig. 5 *E*), demonstrating that REF-52 cells proliferate with normal ploidy.

Flow cytometric analysis of the DNA content demonstrates that the progression of TAG cells toward death is accompanied by a transient population of cells with less than 2N ploidy (Fig. 6 *A*), characteristic of apoptotic cells (Hotz et al., 1994). Extensive death is apparent after treatment with Taxol for either 10 or 40 h, despite the fact that cells treated with Taxol for 10 h had not apparently progressed past G2/M before release.

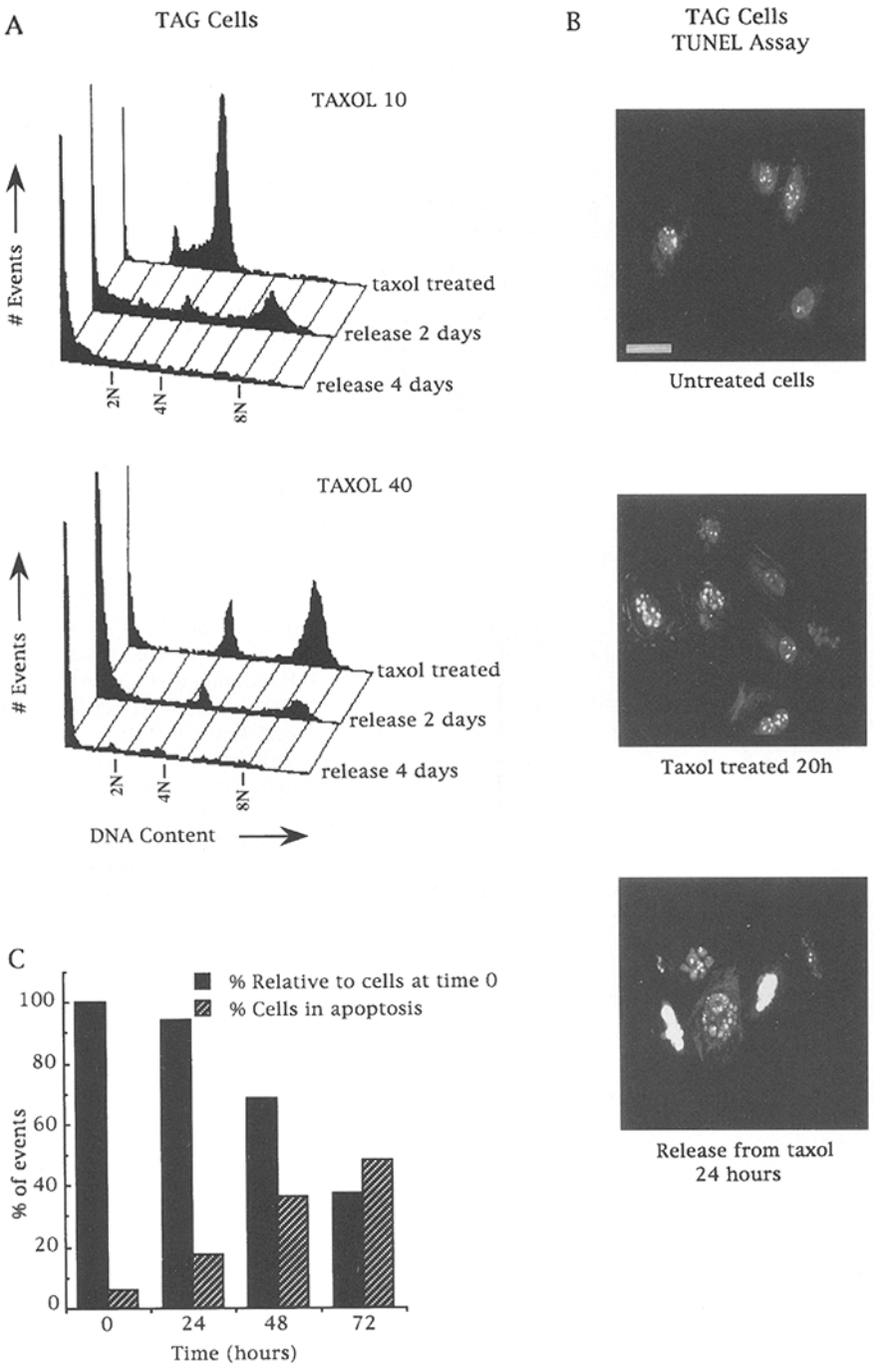

Figure 6. Cell death following Taxol treatment is apoptotic in nature. (*A*) Flow cytometric analysis of TAG cell death following release from Taxol treatment was performed. TAG cells were treated with 5 μM Taxol for 10 or 40 h as indicated. Samples were taken for flow cytometry at the end of the Taxol treatment, or after 2 or 4 d of release, as indicated. (*B*) Microscopic TUNEL assay of untreated TAG cells, or of TAG cells treated with 5 μM Taxol for 20 h, or of TAG cells 24 h after release from 20-h Taxol treatment. Background levels are shown to indicate the presence of non-apoptotic cells. In contrast to this weak background stain, apoptotic cells yield an intense positive signal (*bottom image*). (*C*) Quantification of the extent of apoptosis, as determined by microscopic TUNEL assay. At the time points indicated, representing time following release from 20 h exposure to Taxol, TAG cells were fixed and subjected to the TUNEL assay. The percent of apoptotic cells relative to total cells at each time is shown (*hatched bars*). For reference, the percent of cells still present relative to those at the time of release from Taxol is also shown (*solid bars*). Bar: (*B*) 20 μm.

To confirm that cell death in response to Taxol treatment is apoptotic, we performed a terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) assay, which is indicative of an apoptotic state (Gavrieli et al., 1992; Hotz et al., 1994). There is no positive signal by the TUNEL assay in either control cells or in cells treated with Taxol for 20 h. In contrast, after 24 h release from the drug the assay yields bright nuclear signals in apoptotic cells (Fig. 6 *B*, *bottom image*). The percentage of apoptotic cells, as scored by the TUNEL assay, at varying times after release from 20 h Taxol treatment, are shown in Fig. 6 *C*. For comparison, the number of surviving cells relative to the time of release from Taxol is also shown. In accord with data from flow cytometry (Fig. 6 *A*), the relative extent of apoptosis increases with time following release. These data are in accord with reports that Taxol induced death is apoptotic in nature (Bhalla et al., 1993; Donaldson et al., 1994).

Discussion

We have demonstrated that Taxol imposes a durable late G1 block to cell cycle progression in nontransformed REF-52 and WI-38 cells. We have also shown that SV40 large T antigen transformation of these cells abolishes the Taxol-dependent G1 block point. Finally, we have demon-

strated a dramatic difference in Taxol-induced cell death in transformed and nontransformed cells.

Role of Microtubules in Regulating Cell Cycle Progression

It is clear from our evidence that microtubules are involved in G1 control. But while nontransformed REF-52 cells arrest in G1 in response to Taxol, T antigen–transformed cells do not. From this difference, we can conclude that the G1 arrest point observed in the presence of Taxol is not metabolically necessary to the cell, but it rather appears to result from a microtubule-dependent step in G1 that is absent in transformed cells. Both the regulatory pathways that induce progression through G1 and the G1 checkpoint controls that restrict progression from G1 into S phase can be altered in transformed cells (Donehower et al., 1992; Jacks et al., 1992; Hinds et al., 1994; Mansour et al., 1994). Thus, it is possible that either of these pathways may function in a microtubule-dependent fashion that is specific to nontransformed cells.

In the first case, it may be that a critical element of G1 control is sequestered on microtubules, and that the activation of this element requires its release. The release and activation of this element can occur either in a dynamic microtubule system, or can be triggered in the absence of microtubules, but would not occur when microtubules are completely stabilized by Taxol. In this case, only tumor cells which lack the G1 control would advance to S phase.

Alternatively, by inducing hyperassembly of microtubules, Taxol might impose a checkpoint control in nontransformed cells. Checkpoint controls, which normally function to assure the integrity of the genome, arrest the cell cycle at a discrete point until all prerequisite events have been completed (Hartwell and Weinert, 1989). For example, cell cycle arrest normally follows DNA damage in G1, but this checkpoint control pathway is often ineffective in transformed cells (Kastan et al., 1991; Kuerbitz et al., 1992).

Previous studies have demonstrated that disassembly of microtubules by colchicine and other microtubule assembly inhibitors had effects on cell cycle progression into S phase (McClain and Edelman, 1980; Otto et al., 1981; Crossin and Carney, 1981), but previous results were variable and did not reveal a quantitative arrest in G1 by inhibitors of microtubule dynamics (for review see Otto, 1987). By using flow cytometry and biochemical assays of the components involved in G1 regulation, we have for the first time determined the presence of a stable microtubule-sensitive step late in G1. Also, we demonstrate that transformed cells and their nontransformed counterparts differ in their requirements for dynamic microtubules during progression through G1. Until now the effect of Taxol on interphase progression has not been studied systematically. In the most relevant study to date, colchicine was shown to enhance entry into S phase under serum starvation conditions, while Taxol had an inhibitory effect on colchicine-dependent progression (Crossin and Carney, 1981). In light of these previous observations, it is of interest to note that we find nocodazole, which blocks microtubule assembly in a manner similar to colchicine (Hoebeke et al., 1976), noticeably delays advancement from contact inhibition to S phase (Fig. 3).

The results we have reported here may be compared with our previous observations concerning microtubule control of mitotic exit (Andreassen and Margolis, 1994). In that study we found that the microtubule assembly state determined the capacity of a cell in mitotic arrest to exit mitosis, either through natural "mitotic slippage" or through the effect of the protein kinase inhibitor, 2-aminopurine (2-AP). Both Taxol and nocodazole yielded effective mitotic arrest, but 2-AP was able to induce rapid exit from this blocked state under certain circumstances. 2-AP promoted a rapid exit from Taxol-arrested mitosis, while high nocodazole concentrations prevented checkpoint override and created a stable mitotic arrest. We observed a similar variance in the capacity of Taxol or nocodazole-blocked cells to spontaneously exit mitosis through mitotic slippage (Andreassen and Margolis, 1994).

Our results with mitotic cells and those presented here demonstrate that there are at least two points in the cell cycle where the microtubule assembly state can determine cell cycle progression. However, the effects of Taxol and nocodazole on mitotic exit contrast with their effects on G1 progression. In mitosis, nocodazole can create a stable block to advancement whereas Taxol cannot; while in G1, Taxol creates a stable block to cell cycle advancement where nocodazole does not. The differences in response to the microtubule inhibitors indicate that the mechanisms underlying microtubule effects on mitotic progression and on G1 progression must be different.

The Nature of the Microtubule-dependent G1 Control Point

The two nontransformed cell lines that we have examined, REF-52 and WI-38, arrest stably in late G1 when exposed to Taxol. Contact inhibition is believed to impose a late G1 arrest on mammalian cells (Polyak et al., 1994*a*; Deffie et al., 1995). We have demonstrated that the drug imposed G1 arrest point comes after the point of arrest with contact inhibition. Thus, contact inhibited cells, released into Taxol, exhibit a durable and effective G1 arrest in the presence of the drug.

Late G1 markers present in REF-52 cells at the time of contact inhibition are retained after release into Taxol. Cdk2 protein kinase activity is required to induce the onset of S phase (Fang and Newport, 1991; Pagano et al., 1993; Tsai et al., 1993). Cdk2 activity is negligible both at contact inhibition and in cells arrested with Taxol following contact inhibition. Thus, it appears that activation of cdk2 kinase is inhibited by the presence of Taxol. In contrast, cdk4, which is activated in mid-to-late G1, before activation of cdk2 (Matsushime et al., 1994; Sherr, 1994), shows activity that is equivalent in both contact-inhibited and in G1 Taxol-arrested cells.

$p27^{Kip1}$, a cyclin-dependent kinase inhibitor (Polyak et al., 1994*a*,*b*; Toyoshima and Hunter, 1994) that is specifically degraded in a ubiquitin-dependent pathway during late G1 (Pagano et al., 1995), declines in abundance as the cell enters S phase (Nourse et al., 1994; Halevy et al., 1995). We have found that $p27^{Kip1}$ levels are equivalent in contact-inhibited and in Taxol-arrested cells. Since expression of $p27^{Kip1}$ induces G1 arrest (Polyak et al., 1994*b*; Toyoshima and Hunter, 1994), the continued presence of

$p27^{Kip1}$ may maintain Taxol-dependent G1 arrest by inhibition of cdk2 activity. $p21^{CIP1/WAF1}$, which like $p27^{Kip1}$, is a specific inhibitor of cdk2, is present at diminished levels in Taxol-arrested G1 cells relative to its level in contact-inhibited cells. In response to DNA damage, p53 induces high expression levels of $p21^{CIP1/WAF1}$ in G1 cells (El-Deiry et al., 1994), and $p21^{CIP1/WAF1}$ then suppresses cell cycle progression to S phase (Harper et al., 1993; Deng et al., 1995). Our results thus demonstrate that Taxol does not arrest REF-52 cells in G1 through induction of $p21^{CIP1/WAF1}$. These results are in sharp contrast to the results of Wahl et al. (1996), who report an induction of $p21^{CIP1/WAF1}$ and G1 arrest, subsequent to cell transit through mitosis following a brief treatment with Taxol. Thus, the primary G1 arrest that we observe in response to Taxol results from a molecular mechanism distinct from that reported previously.

Another criterion that indicates that Taxol arrests nontransformed cells in G1, before the onset of S phase, comes from immunolocalization of PCNA. PCNA, a component of the DNA replication complex (Bravo et al., 1987) that localizes to the nucleus throughout S phase (Bravo and MacDonald-Bravo, 1985), is present in the nuclei of REF-52 cells released from contact inhibition into HU, but is excluded from the nuclei of both contact-inhibited cells and cells released from contact inhibition into Taxol.

pRb negatively regulates progression from G1 into S phase. It is unphosphorylated in contact-inhibited cells and through most of G1, but becomes phosphorylated as the cell progresses to S phase (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989). Lack of pRb phosphorylation arrests the cell in late G1, and phosphorylation relieves this inhibition (for review see Hinds and Weinberg, 1994). In cells released from contact inhibition into Taxol, pRb becomes only partially phosphorylated. This change indicates that the cell has measurably progressed from the contact inhibited state to a distinct block point in late G1. Association with pRb inhibits the transcription factor E2F (Hiebert et al., 1992; Weintraub et al., 1992), thereby repressing transcription of several key elements required for progression to S phase. E2F binds the underphosphorylated form of pRb (Chellappan et al., 1991), and cdk-dependent phosphorylation of pRb disrupts the complex and releases the repression of transcriptional activation by E2F (Dynlacht et al., 1994). Both cdk4/cdk6, associated with cyclins of the D class (D1, D2, and D3), and cdk2-cyclin E, have been implicated in the phosphorylation of pRb (for review see Hinds and Weinberg, 1994). Since cdk2 is inactive in Taxol-arrested REF-52 cells, partial phosphorylation of pRb must be mediated by either the cdk4 activity observed, or through cdk6. However, the mechanism underlying the change in the pRb phosphorylation state is currently unknown.

Taxol-dependent arrest in G1 does not occur in TAG cells, which were transformed by introduction of SV40 large T antigen (Perry et al., 1992). The transforming capacity of T antigen has been linked to its distinct effects on G1 control mechanisms. T antigen disrupts several key negative regulators of progression from G1 into S phase. SV40 large T antigen and other viral oncoproteins, bind exclusively to the underphosphorylated form of pRb (Ludlow et al., 1989), thereby sequestering active pRb and inhibiting its growth suppressive function (Hinds et al., 1992). T antigen also binds to p53 (Lane and Crawford, 1979; Linzer and Levine, 1979) and p107 (Dyson et al., 1989), two other proteins that negatively regulate entry into S phase. Interaction of the NH_2-terminal region of T antigen with p53 suppresses p53-mediated growth arrest (Quartin et al., 1994), and has been directly linked to the capacity of T antigen to immortalize primary cells (Zhu et al., 1991).

It is more likely that Taxol-treated cells arrest in G1 due to a microtubule-dependent checkpoint control, than that they fail to progress from contact inhibition due to an indirect effect of the loss of microtubule organization on the function of the signal transduction machinery. If this were true, one would expect a similar effect with nocodazole, since nocodazole disrupts the microtubule cytoskeleton more profoundly than Taxol. However, we have shown that nocodazole does not induce a stable G1 block to cell cycle progression.

One can assume that some components of the machinery necessary for G1 progression in nontransformed cells must associate with microtubules. The results we have presented here should make a search for microtubule-associated elements involved in G1 control worthwhile, and we are currently pursuing this direction of inquiry. We are also currently working to determine the mechanism by which Taxol interferes with cdk2 activation and pRb phosphorylation during G1.

Implications for Tumor Therapy

Different microtubule inhibitors vary in their ability to selectively destroy tumor cells in a clinical setting. While the chemotherapeutic effect of microtubule inhibitors would seem in some way linked to the most evident effect of the drugs, mitotic arrest, there is no direct evidence in support of this assumption. Both Taxol and nocodazole impose mitotic arrest on mammalian cells. Taxol has shown promise in chemotherapy, particularly in the treatment of ovarian and breast tumors (Rowinsky and Donehower, 1995), while nocodazole has not. The difference in chemotherapeutic efficacy may lie in two more subtle effects in the cell cycle. We have shown that Taxol can induce a rapid mitotic slippage, whereas higher concentrations of nocodazole create a stable mitotic block (Andreassen and Margolis, 1994). Furthermore, as we have shown here, Taxol produces a stable G1 block that selectively occurs in nontransformed fibroblast cells, while nocodazole does not induce G1 arrest. We are currently working to determine if similar effects occur in nonfibroblast cell lines.

We have shown elsewhere that Taxol enhances mitotic slippage (Andreassen and Margolis, 1994), and have shown here that transformed TAG cells undergo mitotic slippage with Taxol, while nontransformed REF-52 cells do not (Fig. 1). We have observed similar differences in mitotic slippage between WI-38 and VA-13 cells (our unpublished observations). It is therefore possible that mitotic slippage in Taxol may produce selective lethality in tumor cells, and that failure to arrest in G1 ultimately exposes the entire transformed cell population to mitotic slippage.

The lethal event may also occur on passage from G1 to S in Taxol, since our results have indicated that cell death is

induced somewhere between mitotic exit and the onset of S phase. Other recent studies have suggested that Taxol may have a lethal effect early in the cell cycle (Donaldson et al., 1994; Hennequin et al., 1995). The early cell cycle effect is even more striking for a Taxol-related drug, taxotere, and it has been suggested that lethality may be related to inhibition of centrosome duplication during interphase (Hennequin et al., 1995).

We show that Taxol is not lethal to TAG cells if they are exposed to the drug during the period of the cell cycle between early S phase and exit from mitosis. These conclusions were drawn from an experiment where TAG cells were either exposed to Taxol alone or were released from HU into Taxol. Cells released from HU and then exposed to Taxol during the period of the cell cycle between early S phase and exit from mitosis experienced no lethal effect, whereas exposure of a randomly cycling, and mostly G1, population to Taxol for an equivalent period of time yielded substantial lethality.

In addition to raising some very interesting biological questions concerning the capacity of the microtubule assembly state to determine G1 progression, our results may suggest a unique target for tumor chemotherapy. It is possible that a screen of drugs that interfere with microtubule dynamics for a subset differentially capable of arresting nontransformed cells in G1 will yield a unique class of compounds with unusual selectivity for tumor cells.

We thank Dr. Rati Fotedar (I.B.S.) for cdk2 antibody, and Dr. George R. Stark (Cleveland Clinic Foundation) for providing REF-52 and TAG cell lines.

This work was supported in part by grants from the International Human Frontier of Science program, and from the Association pour la Recherche contre le Cancer (ARC). Marc Trielli is supported by a fellowship from La Ligue Nationale contre le Cancer. This is publication No. 423 of the Institut de Biologie Structurale Jedn-Pierre Ebel.

Received for publication 26 March 1996 and in revised form 12 August 1996.

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