Radiosensitivity of Thymidylate Synthase-deficient Human Tumor Cells Is Affected by Progression through the G$_1$ Restriction Point into S-Phase: Implications for Fluoropyrimidine Radiosensitization

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ABSTRACT

Recent studies of fluoropyrimidine (FP)-mediated radiosensitization (RS) have focused on the molecular mechanisms underlying regulation of the cell cycle, particularly at the G$_1$-S transition. Although thymidylate synthase (TS) inhibition by FP is necessary, we hypothesize that FP-RS is temporally dependent on progression of cells into S-phase under conditions of altered deoxynucleotide triphosphate pools, particularly an increased dATP:dTTP ratio, which subsequently results in enhanced DNA fragmentation and cell death. To better understand the mechanism of FP-RS, we characterized the cellular and biochemical responses to ionizing radiation (IR) alone, using different synchronization techniques in two isogenic, TS-deficient mutant cell lines, JH-1 (TS$^+$) and JH-2 (Thr4), derived previously from a human colon cancer cell line. After G$_0$ synchronization by leucine deprivation, these clones differ under subsequent growth conditions and dThd withdrawal: JH-2 cells have an intact G$_1$ arrest (>72 h) and delayed cell death (>96 h), whereas JH-1 cells progress rapidly into early S-phase and undergo acute cell death (<24 h). No difference in the late S-phase and G$_2$/M cell populations were noted between these growth-stimulated, G$_0$-synchronized TS-deficient cell lines with dThd withdrawal. Biochemically, the intracellular ratio of dATP:dTTP increased substantially in JH-1 cells as cells progressed into early S-phase compared with JH-2 cells, which remained in G$_0$ phase. Synchronized JH-1 cells showed significantly decreased clonogenic survival and an increase in DNA fragmentation after IR when compared with JH-2 cells. RS was demonstrated by an increase in $\alpha$ and decrease in $\beta$, using linear quadratic analyses. An alternative synchronization technique used mimosine to induce a block in late G$_0$, close to G$_1$-S border. Both JH-1 and JH-2 cells, synchronized in late G$_0$ and following growth stimulation, now progressed into S-phase identically (<24 h), with similarly increased dATP:dTTP ratios under dThd withdrawal conditions. These late G$_0$-synchronized JH-1 and JH-2 cells also showed a comparable reduction in clonogenic survival and similar patterns of increased DNA fragmentation following IR. We suggest, based on the cellular and biochemical differences in response to IR between G$_0$- and late G$_0$-synchronized cells, that S-phase progression through the G$_0$ restriction point under an altered (increased) dATP:dTTP ratio is a major determinant of FP-RS.

INTRODUCTION

The FPs, including 5-FUra and FdUrd, are widely used as effective clinical radiosensitizers in many human cancers, particularly those arising from the gastrointestinal tract (1, 2). A major mechanism of FP cytotoxicity is inhibition of TS by fluoro-dUMP, their common metabolite (3, 4). Prolonged TS inhibition results in an alteration of cellular nucleotide pools (especially dITTP depletion; Ref. 5), changes in cell cycle distribution (6, 7), and induction of DNA strand breakage/fragmentation with subsequent clonogenic cell death (5, 8, 9).

Studies assessing the value of combining FP with IR commenced soon after their synthesis in the late 1950s (1, 2). Experimental studies in mice with sarcoma 180 tumors demonstrated significant tumor responses with the combination of i.p. 5-FUra followed by a single dose of IR, which was not found with i.p. 5-FUra alone (10). Approximately a decade later, clinical investigators reported the first prospective trial in unresectable gastric cancer in which they found a modest survival benefit to intermittent low-dose bolus infusion 5-FUra during IR (11). A similar intermittent, low-dose bolus schedule of 5-FUra during IR was found to be effective as a radiosensitizing regimen in resected rectal and pancreatic cancers in the Gastrointestinal Tumor Study Group randomized trials performed in the 1980s (12–14). Because the low-dose schedule of 5-FUra would not be expected to result in significant tumor cytotoxicity, most clinical investigators have interpreted these data to suggest a role for 5-FUra as a radiosensitizer. More recently, combined modality studies in resected stage II and III rectal cancer and in locally advanced esophageal cancer have shown a further survival advantage to the use of longer infusion 5-FUra compared with higher dose bolus infusion during IR (15–17). Indeed, the combination of 5-FUra and IR as a postoperative adjuvant is now the standard of care for stage II and III rectal cancers as determined by a NIH consensus conference (18).

Despite the many positive clinical trials in gastrointestinal cancers, the biochemical and molecular mechanisms of interaction of FP and IR remain to be further clarified in the laboratory (1, 2). Indeed, the usual flow of ideas and information from laboratory to the clinic has been reversed for FP-RS until quite recently. In the 1980s, conflicting laboratory data were reported, with some in vitro data suggesting that greater than additive effects were found in human tumor cell lines only with post-IR exposure using cytotoxic doses of 5-FUra (19, 20), whereas in vivo laboratory studies suggested only additive effects with the combination of 5-FUra and IR, with primary dependence on the total dose of 5-FUra but not on the mode nor schedule of drug administration (21, 22). As a consequence of these reports, current clinical trials of this combined approach designed in the late 1980s and early 1990s are based on enhancing 5-FUra cytotoxic effects using either biochemical modulators, as found in the adjuvant rectal cancer studies, or additional cytotoxic drugs such as cis-platinum, which may also interact with 5-FUra and/or IR, as was found in clinical trials of esophageal and anal cancers. Thus, it is not clear whether these clinical trials are addressing RS or simply additive drug-IR cytotoxic effects. Unfortunately, the design of these ongoing clinical trials will not address this concern, which underscores the need for additional preclinical studies that may have an impact on mechanism-based clinical trials of FP-RS in the near future.

Recent laboratory investigations point to biochemical and molecular events at the G$_1$-S interface as potentially important determinants of subsequent FP-RS as cells proceed into S-phase (23–30). Our group and investigators at the University of Michigan were the first to
demonstrate that the use of minimally cytotoxic doses of FdUrd prior to IR in human colorectal cell lines results in an immediate inhibition of TS (<5% TS activity within 1 h) followed by a later expansion of an early S-phase tumor cell population that correlates temporally with enhanced in vitro RS (23–25). FdUrd was used in these in vitro studies, instead of 5-FUra, to limit any additional effect of FP on RNA and protein synthesis. Both groups also subsequently found that enhanced RS of an enriched early S- to mid-S-phase tumor cell population was not seen when cell synchronization techniques alone were used (25, 26). Additionally, prior exposure to FP results in a decrease in sublethal damage repair as well as a decrease in the repair of DNA single-strand breaks and double-strand breaks after IR (27, 28). However, FP-RS does not result in an increase in initial DNA damage, in contrast to RS using the halogenated dThd analogues (1).

MATERIALS AND METHODS

Materials. BUdR, Tween 20, PI, antimonous IgG-FITC, pepsin, BSA, RNase A, and normal goat serum were obtained from Sigma Chemical Co (St. Louis, MO). DNA size markers and InCert agarose were obtained from FMC BioProducts (Rockland, ME), and low endoosmosis agarose was purchased from Fisher Scientific (Fair Lawn, NJ). Mimosine (Sigma) stock solution was resuspended in culture medium at a 5 mM concentration. All other reagents were of the highest grade available.

Cell Culture and Synchronization. The JH-1 cell line, a TS-deficient mutant cell line, was isolated from parental GC/c1 human colon carcinoma cells and had previously been named TS− (31). The JH-2 (previously named Thy4) cell line was isolated from the JH-1 line as apoptosis-resistant cells following repeated rounds of dThd withdrawal (31). Both cell lines have similar doubling times (30 h) and show ≤5% TS activity levels compared with the parental GC/c1 control cells (data not shown). These cells were cultured in RPMI 1640 (Life Technologies) containing 10% dialyzed fetal bovine serum, 712 μM CaCl2, and 20 μM dThd. In all experiments, cells were plated and then incubated overnight before each assay. Cells were synchronized in G0 by leucine deprivation for 4 days, as described previously (32, 33). To synchronize cells in late G1, cells were treated with 300 μM mimosine for 22 h. After both synchronization techniques, fresh medium was added and cells were harvested at various times. The other culture conditions of the two TS-deficient cell lines have been described previously (31–35).

Analysis of dNTP Pool Levels. The preparation of cell extracts and HPLC conditions for dNTP pool measurements were performed according to Tanaka et al. (38). Cells in exponential growth were detached by trypsinization, washed twice with PBS, and suspended at 4°C in 100 μl of PBS. After the cells were counted, cold 100% trichloroacetic acid was added to the final concentration of 0.3 M. The mixtures were subjected to repeated cycles of vortexing and cooling on ice for 30 min, and then centrifuged (14,000 rpm, 1 min, 4°C). The acid supernatant was recovered and neutralized by adding 1.1 volumes of cold Freon-amime solution (0.5 M tri-n-octylamine in 1.12-trichloro-trifluoroethane), and mixed (2-min vortex). The liquid phase was separated by centrifugation (14,000 rpm, 1 min, 4°C), and the aqueous upper layer (called the cell extract) containing the nucleotides was recovered. For quantitative determination of dNTP pools, the cell extract was treated with periodate and methylamine to decompose the ribonucleotides. Fifteen μl of 20 mM deoxyguanosine and 15 μl of 0.2 M NaIO4 were added to 60 μl of cell extract. The mixtures were incubated at 37°C for 5 min, and cooled on ice. Two μl of 1 M l-threonine and 9 μl of 4 M methylamine (neutralized to pH 6.5 with H3PO4) were then added, incubated at 37°C for 30 min, and then cooled. This final mixture was analyzed using a Waters HPLC system (600E multisolvent delivery system and controller, 490E multiwavelength detector, 717 autosampler, and Millenium chromatography manager software).

Nucleotides were separated on a 4.6 × 250 mm Partisil-10 SAX column (Whatman). The mobile phase consisted of 0.35 M (NH4)2HPO4 (pH 3.0) with H3PO4 at a flow rate of 2 ml/min. Peaks were detected at 254 nm. The retention times of dCTP, dGTP, dATP, and dGTP were 10.6, 12.4, 14.5, and 26.8 min, respectively. dNTPs were quantified by peak heights against authentic standards using the Millenium software.

Measurement of Apoptotic Cell Death. After the various cell treatment protocols, cells were collected by trypsinization and washed with Cal-Mg2+-free PBS. For measurement of apoptotic cells, both attached and nonadherent cells were collected and stained with PI according to Darzynkiewicz et al. (39), and analyzed by flow cytometry as described below.

Cell Cycle Distribution Analysis Using Flow Cytometry Analysis. Samples for cell cycle distribution analysis were prepared using a modification of the technique by Schutte et al. (40). Cells were analyzed by two-parameter flow cytometry measuring PI fluorescence and fluorescence of FITC-conjugated goat antioimmune antibody against mouse anti-BUdR primary antibody as follows. After dThd withdrawal, cells were pulse-labeled with 20 μM BUdR for 15 min prior to harvesting for each time point. Dishes were then washed twice with PBS, resuspended by trypsinization, and centrifuged (300 × g, 5 min, 4°C). Approximately 2 × 106 cells were used for each sample. Cells were then resuspended in cold PBS (0.7 ml), fixed in 95% ethanol containing 0.5% Tween 20 (1.3 ml), and then stored at 4°C until sample collection was completed (up to 7 days). After fixation, 5 ml of PBS were added, and the cells were again centrifuged (300 × g, 5 min). The pellet was resuspended in a solution of 0.5 ml of 0.04% pepsin in 0.1 N HCl and incubated for 30 min at room temperature. The nuclei were pelleted (600 × g, 5 min), gently resuspended in 1.5 ml of 2 N HCl, and then incubated for 30 min at 37°C. Nuclei were washed twice with 5 ml of PBS-TB (PBS containing 0.5% Tween 20 and 0.1% BSA), resuspended in 1 ml of RNase A (10 μg/ml in PBS-TB), and then incubated in the dark for 20 min at 37°C. RNase A was removed by centrifugation (600 × g, 5 min) and the nuclei were washed with 3 ml of PBS-TB.
The nuclei were then resuspended in 50 μl of PBS-TB and 20 μl of anti-BUdR antibody (Becton Dickinson, San Jose, CA) and incubated at room temperature in the dark for 90 min. Three ml of PBS-TB were added to each sample and centrifuged (600 × g, 5 min). The pellet was resuspended using 0.2 ml of antimouse IgG-FITC at a 1:50 dilution in PBS containing 0.5% Tween 20 and 0.1% goat serum (Sigma) and incubated for 20 min at room temperature in the dark. The nuclei were washed twice in 3 ml of PBS-TB, followed by incubation with 50 μg/ml PI in 0.5 ml of PBS-TB. Cells were filtered through nylon mesh (Nitex; Tetko Inc., Briarcliff Manor, NY), and then analyzed under dual-parameter (FITC compared with PI) conditions with a Becton Dickinson FACScan using Cell Quest data acquisition. Doublets were excluded by gating the dot plots of fluorescence pulse width versus area of PI signal, ensuring that only singlets were analyzed for cell cycle distribution statistics. Windows were drawn to define cell populations in each cell cycle phase fraction in contour plots of FL1 (FITC signal) versus FL3 (PI signal). The population with the BUdR-negative signal (unlabeled populations) and 2N DNA content detected by PI fluorescence were defined as G0-G1, and 4N DNA content cells were defined as G2-M. BUdR-positive cells (pulse-labeled with BUdR) were defined as S-phase cells. To determine the cell fraction in early or late S-phase, the S-phase region of PI fluorescence was divided in half between 2N and 4N DNA. Cells within the sector with less PI signal were defined as the early S-phase population, and those within the sector with higher PI staining were defined as the late S-phase population. Cell cycle fractions were calculated using Cell Quest software.

Clonogenic Survival Assay after IR. Exponentially growing cells were irradiated using a 137Cs source at a dose rate 5.927 Gy min⁻¹. The synchronized cells were placed in dThd-deficient medium for 0, 24, or 48 h prior to IR and then placed in medium containing 20 μM dThd. Asynchronous populations of JH-1 and JH-2 cells were also subjected to dThd withdrawal plus IR and rescued by dThd (20 μM) at the same time points. For each time point, the cell suspension was distributed into five 25-cm² flasks, which were put on ice until irradiation. The flasks were irradiated (0–4 Gy) at room temperature and then were plated (in triplicate) in dishes (60-mm) containing 5 ml of medium. The dishes were incubated for 15 days in an incubator at 37°C in a humid 5% CO2 atmosphere. Surviving colonies (>50 cells/colony) were stained and counted. Clonogenic survival values were derived from at least three independent experiments.

dThd Withdrawal IR Survival Analysis. We have described a two-stage method for analysis of cell survival sequences (41). Each dose-response sequence is first fitted to a linear-quadratic model, and then estimated parameters of α and β are determined.

DNA Damage Detection by PFGE. The induction of DNA double-strand breaks in asynchronous and both G0- and late G1-synchronized cell populations after dThd withdrawal ± IR was determined by PFGE as follows. Whole-cell populations were collected by trypsinization and washed in Ca²⁺-Mg²⁺-free PBS, and the cell number was determined. The 2% agarose (InCert Gel agarose) solution was prepared in 0.5× TBE (0.45 M Tris-borate, 0.01 M EDTA) buffer, maintained at 50°C. Cells were mixed with the agarose solution
at a concentration of $1 \times 10^7$ cells per ml of agarose block. The agarose-cell suspension was then poured into molds and placed on ice. Cells embedded in agarose were lysed in a solution containing 100 mM EDTA (pH 8.0), 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, and 1 mg/ml proteinase K for at least 24 h at 50°C. The plugs were then washed four times in wash buffer (20 mM Tris, pH 8.0, 50 mM EDTA) at room temperature. The DNA agarose plugs were stored in 0.1× wash buffer at 4°C until PFGE analysis. PFGE was performed using a Bio-Rad (Richmond, CA) CHEF-DRII apparatus with an electric field reorientation angle of 120 degrees. The plugs were inserted into 0.7% gels made from low endosmosis agarose in 0.5× TBE. Electrophoresis was performed at 14°C in 0.5× TBE in two stages. The first stage was performed for 30 h, using a linearly increasing pulse time gradient from 30 to 120 s with a field strength of 1.9 V/cm. The second stage was performed for 51 h, using a linearly increasing pulse time gradient from 2 to 42 min with a field strength of 1.9 V/cm. Yeast chromosomes from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were included in each gel to calibrate the DNA fragment size. After electrophoresis, the gels were stained for 30 min in 0.5 μg/ml ethidium bromide solution and destained for 3 h in distilled water. The gels were scanned using a Fluoromager (Molecular Dynamics, Inc., Sunnyvale, CA).

**RESULTS**

Clonogenic Survival and Apoptotic Fraction of $G_0$-synchronized and Asynchronously Growing Cells after dThd Withdrawal. To characterize the cell lines, we began these studies by comparing the cytotoxic effect of dThd withdrawal on JH-1 and JH-2 cells. We examined clonogenic survival of asynchronous and $G_0$-synchronized cells after release from dThd withdrawal at 24-h intervals for up to 144 h (6 days; Fig. 1, a and b). In asynchronous populations, survival of JH-1 and JH-2 cells was similarly decreased after dThd withdrawal. Less than 10% of either cell line survived after 96 h of dThd withdrawal. In contrast, whereas $G_0$-synchronized JH-1 cells showed loss of survival under dThd withdrawal similar to that seen with both asynchronous populations, $G_0$-synchronized JH-2 cells maintained 85% viability for at least 96 h. At 144 h, JH-2 cells still maintained 55% viability. These results are consistent with previous observations (31, 32).

We also measured the apoptotic population using flow cytometry. As shown in Fig. 1c, in asynchronous growth, both JH-1 and JH-2 cells accumulated an apoptotic population after dThd withdrawal. By 120 h, the apoptotic (sub-$G_0$) population constituted ~38% within both cell types. $G_0$-synchronized JH-1 cells also show the accumulation of an apoptotic population after dThd withdrawal. However, $G_0$-synchronized JH-2 cells showed less of an apoptotic population compared with asynchronous and $G_0$-synchronized JH-1 cells (10% compared with 38% at 120 h; Fig. 1d). Consistent with previous reports (31, 32), synchronized or asynchronous JH-1 cells and asynchronous JH-2 cells undergo dThd withdrawal-induced acute apoptosis. In contrast, $G_0$-synchronized JH-2 cells survived (85%) for up to 96 h under dThd withdrawal (Fig. 1).

Changes in dNTP Pools of $G_0$-synchronized and Asynchronously Growing Cells after dThd Withdrawal. dNTP pools were examined in asynchronously growing JH-1 and JH-2 cell populations for 48 h under dThd withdrawal. The dNTP pools of both asynchronously growing cell populations changed similarly; dTTP and dGTP decreased immediately, whereas dATP levels increased within 6 h of dThd withdrawal. The imbalance of these dNTP pools reached its maximum at 18–24 h. In contrast, dCTP showed no significant change over the 48-h period. dGTP levels were very low in untreated cells and were below quantifiable levels within 6 h after dThd withdrawal. The observed pattern of dNTP pool imbalance is similar to previously published data in asynchronously growing JH-1 cells (33) and to FdUrd-treated FM3A mouse mammary tumor cells (5).

The most significant effects on dNTP pools involved dTTP and dATP levels. In Fig. 2a, the change in dATP:dTTP ratios in asynchronously growing JH-1 and JH-2 cells populations after dThd withdrawal are shown. We standardized the initial dATP:dTTP ratio of each cell line to 1. In asynchronous JH-1 cells, the initial levels of dATP and dTTP were $9.2 \pm 1.41$ and $24.6 \pm 3.39$ pmol/10^6 cells, respectively, and the initial levels in asynchronous JH-2 cells were $12.1 \pm 2.05$ and $38.3 \pm 5.80$ pmol/10^6 cells, respectively. The dATP:dTTP ratio in asynchronous populations of both JH-1 and JH-2 cells increased ~15-fold (14.7- and 15.9-fold, respectively) within 24 h of dThd withdrawal (Fig. 2a). We also examined dNTP pools in $G_0$-synchronized JH-1 and JH-2 cells following release and under dThd withdrawal (Fig. 2b). After 24 h, $G_0$-synchronized JH-1 cells showed a 5-fold increase in the dATP:dTTP ratio, which increased to 15-fold at 72 h. In contrast, $G_0$-synchronized JH-2 cells showed an initial 4-fold increase at 24 h but no further significant change in the dATP:dTTP ratio over the 96-h period of measurement.

Cell Cycle Distribution of $G_0$-synchronized and Asynchronously Growing Cells after dThd Withdrawal. As discussed previously, recent *in vitro* studies using HT-29 human colon cancer cells have suggested that cell cycle progression through the $G_1$-$S$ checkpoint may be involved in determining the extent of FP-RS...
To assess whether this G1-S checkpoint was intact in TS-deficient cells under dThd withdrawal, we examined the cell cycle distribution of asynchronous and G0-synchronized JH-1 and JH-2 cells after dThd withdrawal, using two-parameter flow cytometry. Fig. 3 shows the percentage of each cell cycle phase from 0 to 120 h. In asynchronously growing JH-1 and JH-2 cells, the G0-G1 population decreased after 24 h of dThd withdrawal, and the early S-phase population increased (Fig. 3, a and c). We also quantitated the cell cycle distribution of G0-synchronized JH-1 and JH-2 cells under dThd withdrawal (Fig. 3, b and d). The G0-G1 population of G0-synchronized JH-1 cells (Fig. 3b) decreased at 24 h (from 71% to 24%) and the early S-phase population increased (from 6% to 53%) to the levels observed in the asynchronous populations of both cell lines after dThd withdrawal (Fig. 3, a and c). However, in G0-synchronized JH-2 cells (Fig. 3d), the G0-G1 population slowly decreased by 24 h (from 71% to 57%), and remained steady at ~40% out to 72–120 h, whereas the early S-phase population increased modestly at 24 h (from 5% to 19%). The S-phase increase continued up to 72 h (44%) and then remained steady from 72–120 h, even when delayed cell death was initially observed (96 h; Fig. 1b). These data suggest that G0-synchronized JH-2 cells appear to arrest in G1 after dThd withdrawal, whereas G0-synchronized JH-1 cells progress into S-phase after dThd withdrawal, similar to the asynchronous populations. No significant differences were seen for the G2-M populations of JH-1 and JH-2 cells (Fig. 3).

**IR Survival of G0-synchronized and Asynchronously Growing Cells after dThd Withdrawal.** We next compared the radiosensitivity using clonogenic survival assays of JH-1 and JH-2 cells in asynchronous growth conditions with dThd withdrawal versus G0-synchronized cells stimulated to grow (leucine added) under dThd withdrawal. Clonogenic assays were repeated at least three times and analyzed by changes in α and β values of the linear-quadratic model as described (41). The IR survival of asynchronous and G0-synchronized cell populations were determined at 0, 24, and 48 h of dThd withdrawal.

There were no significant differences in α and β values between asynchronously growing JH-1 and JH-2 cells (data not shown) and G0-synchronized JH-1 cells under dThd withdrawal for 0, 24, or 48 h prior to IR (Fig. 4). These populations demonstrated a progressive increase in radiosensitivity as measured by both an increase in α and a decrease in β under dThd withdrawal conditions over 48 h (Fig. 4). In contrast, the G0-synchronized JH-2 cells showed no change in α or β when kept under dThd withdrawal conditions for 0–48 h prior to IR (Fig. 4).
DNA Damage Detection in G0-synchronized and Asynchronously Growing Cells after dThd Withdrawal (+ IR) by PFGE.

Induction of DNA damage was determined by PFGE as described. DNA fragmentation occurred in a time-dependent manner over 24–72 h of dThd withdrawal alone in asynchronous JH-1 and JH-2 cell populations (data not shown). A similar increase in DNA fragmentation was also observed in G0-synchronized JH-1 cells with dThd withdrawal alone. In contrast, the rate of DNA fragmentation in G0-synchronized JH-2 cells was significantly reduced (data not shown). These DNA fragmentation patterns correlate with the survival curves and extent of apoptotic fraction in the respective cell populations as detailed in Fig. 1.

We also examined the effects of IR (4 Gy) in G0-synchronized JH-1 and JH-2 cells under dThd withdrawal conditions (Fig. 5a). For these experiments, G0-synchronized cell populations were subjected to dThd withdrawal for 24 h and then irradiated. DNA fragmentation was assessed by PFGE immediately after IR (Fig. 5, Lanes -T/IR) or after 24 h in the presence of medium containing 20 μM dThd (Fig. 5, Lanes -T/IR/+T). Immediately after IR exposure, a major band of 6–9 Mb was observed in both cell populations. This band was not seen after dThd withdrawal for 24 h alone as described above. Additionally, when G0-synchronized cells were incubated with dThd-containing medium for 24 h after IR (Fig. 5, Lanes -T/IR/+T), greater DNA fragmentation was seen in G0-synchronized JH-1 cells versus JH-2 cells. These PFGE data are consistent with the IR survival analysis in Fig. 4.

Cell Cycle Distribution in Late G1-synchronized Cells. We also characterized the cell cycle distribution of JH-1 and JH-2 cells after mimosine synchronization and subsequent dThd withdrawal (Fig. 6). After mimosine treatment, 66.3 and 68.3% of JH-1 and JH-2 cells, respectively, were in G1 phase. After mimosine release and dThd withdrawal, the G1 populations of the JH-1 and JH-2 cells decreased dramatically at 24 h (8.5 and 5.2%), whereas the early S-phase increased (from 0.3 and 2.8% to 54.9 and 63.7% at 24–48 h, respectively) and then decreased slowly.

Comparison of dNTP Pools and IR Survival in G0 versus Late G1-synchronized Cells. Late G1-synchronized JH-1 and JH-2 cell populations showed an immediate (within 6 h of dThd withdrawal) increase in dATP levels (1.0 and 1.5 pmol/10^6 cells to 16 and 36 pmol/10^6 cells, respectively) with peak dATP levels observed after 48 h of dThd withdrawal (57.7 and 56.5 pmol/10^6 cells, respectively). The dTTP levels in both late G1-synchronized cell populations showed an immediate and prolonged decrease, with the calculated dATP:dTTP ratios being quite similar under dThd withdrawal condi-
tions to both asynchronously growing JH-1 and JH-2 cell populations and to G₀-synchronized JH-1 cells (Fig. 2). Interestingly, late G₁-synchronized JH-2 cells under dThd withdrawal also showed a marked increase in the dATP:dTTP ratio (up to 30–40-fold at 24–48 h) compared with a <5-fold increase in G₀-synchronized JH-2 cells (Fig. 7a). These late G₁-synchronized JH-2 cells also showed significantly enhanced radiosensitivity at 24 (Fig. 7b) and 48 h after dThd withdrawal compared with G₀-synchronized JH-2 cells. The calculated α and β values for late G₁-synchronized JH-2 cells after dThd withdrawal for 24 h were 2.04 ± 0.05 and 0.059 ± 0.018, respectively. Additionally, these late G₁-synchronized JH-2 cells showed enhanced IR-related (4 Gy) DNA fragmentation (Fig. 5b) similar to G₀-synchronized JH-1 cells under dThd withdrawal (Fig. 5a).

**DISCUSSION**

In this study, we have presented data consistent with the hypothesis that FP-RS has some common features with the process of thymineless death by comparing the cellular and biochemical responses to IR in two TS-deficient human tumor cell lines (JH-1 and JH-2). We found that, under dThd withdrawal, G₀-synchronized JH-1 cells showed a dramatic increase in the dATP:dTTP ratio, progressed into S-phase, and underwent acute cell death. After IR, JH-1 cells showed further increased cytotoxicity and increased DNA fragmentation. In contrast, G₀-synchronized JH-2 cells showed no significant change in the dATP:dTTP ratio, arrested in G₁, and maintained 55% cell viability until 144 h. These G₀-synchronized JH-2 cells also showed less IR-related DNA fragmentation and reduced radiosensitivity after dThd withdrawal compared with JH-1 cells. However, mimosine-synchronized (late G₁) JH-1 and JH-2 cells progressed into S-phase immediately with increased dATP:dTTP ratios and showed similar enhanced radiosensitivity and enhanced IR-related DNA fragmentation after dThd withdrawal.

Thymineless death was first observed in *Escherichia coli* by Cohen and Barner (42) 40 years ago. More recently, this phenomenon was also reported in mammalian cells by Ayusawa et al. (43), when they isolated a TS-deficient mutant cell from mouse tumor cells. They found that dThd withdrawal induced dNTP imbalances; i.e., depletion of dTTP and dGTP and an increase in dATP levels (5). They also found that dThd withdrawal produces chromosomal strand breaks that paralleled the progression of cell death (44). FdUrd treatment of cells...
also resulted in dNTP imbalance-induced DNA strand breaks and subsequent cell death (5). However, it has been suggested from results using aphidicolin-synchronized cells that FdUrd-induced cell death requires cells to pass through the S-phase (5).

This pattern is quite similar to our results. In the process of acute cell death in our TS-deficient mutants under dThd withdrawal, dTTP levels decreased and dATP levels increased immediately, and these pool imbalances persisted as the cells progressed into S-phase. Furthermore, after IR, we hypothesize that cells that have progressed into the S-phase with marked dNTP imbalances (asynchronously growing and late G1-synchronized JH-1 and JH-2 cells and G0-synchronized JH-1 cells) would be less capable of repairing IR damage. In contrast, G0-synchronized JH-2 cells remained in G1 and maintained a normal dATP:dTTP ratio, resulting in less DNA fragmentation and significantly delayed cytotoxicity. We speculate that the observed reduced cytotoxicity associated with a G1 arrest allows for more complete repair of IR damage before entering S-phase and that this could also explain the observed resistance to thymineless death. It is known that dNTP-pool imbalances can have profound effects on the accuracy of DNA replication in S-phase because dNTP pool sizes at replication forks closely mirror the total dNTP pools (45, 46). It is likely that the dNTP pool imbalances trigger the cellular events leading to thymineless death (dThd withdrawal) of TS-deficient cells and to the enhanced cytotoxicity (RS) of FP-treated cells with IR.

To test this hypothesis, we initiated studies of cell cycle-related RS that focused on the G1-S checkpoint. We synchronized cells in late G1 prior to the G1-S border to determine whether JH-1 and JH-2 cells would respond similarly to IR if synchronized beyond the G1 restriction point. After mimosine synchronization, JH-1 and JH-2 cells progressed into S-phase with an increased dATP:dTTP ratio identically and also demonstrated the same IR response (clonogenic cell survival and DNA fragmentation pattern) under dThd withdrawal. Mimosine was originally proposed to inhibit cells in late G1 (36, 37), although a few other studies have reported that mimosine blocks cells during S-phase (47, 48). However, our flow cytometry data and other data on mimosine effects (36, 37, 49) indicate that mimosine inhibits in late G1-phase, prior to the G1-S border. Therefore, we conclude these two TS-deficient cell lines when synchronized beyond the G1 restriction point by mimosine show similar S-phase progression and similar cell death processes under dThd withdrawal with or without IR.

Numerous studies indicate that the intrinsic sensitivity of mammalian cells to IR is a function of their position in the cell cycle (50–60). In some cases, cells in G1, or at the G1-S border have been reported to be more sensitive to IR than those in other parts of the cycle (50–54). In addition, IR-induced G1 arrest and apoptosis in mammalian cell lines have been shown to require wild-type p53 expression, whereas mutant p53 or loss of p53 function has correlated with reduced cytotoxicity after IR (50–54). Similarly, some data suggest that p53-dependent G1 arrest does not reduce the occurrence of chromosomal aberrations or DNA damage (55, 56). However, others argue that G1 arrest might be important to allow DNA repair prior to replication. One group has suggested that G1 arrest represents activation of a cell cycle checkpoint and is dependent on the presence of functional wild-type p53 protein (57–60). They demonstrated that p53 helps maintain genetic stability by preventing replication of damaged DNA through a prolonged G1 arrest. Similarly, p21 deficiency is associated with defective DNA repair, which could lead to an increased sensitivity of tumor cells to DNA damage/mutation (61).

Both JH-1 and JH-2 cells are reported to be heterogeneous for p53 expression (34). The wt/p53 phenotype correlated with acute apoptosis following dThd withdrawal, whereas the mp3/p53 phenotype was associated with delayed apoptosis. However, expression of p21 did not correlate with either acute or delayed apoptosis after dThd withdrawal (34). In addition, JH-2 cells are capable of sustaining elevated levels of both Bax and Bcl-2, which have been implicated in induction or protection from apoptosis, respectively (34). More recently, it has also been shown that Fas-Fasl interaction is responsible for acute apoptosis in JH-1 cells (35). However, the molecular mechanism responsible for the different responses by JH-1 and JH-2 to dThd withdrawal with or without IR are not clear, and the question still remains as to the relationship between p53, p53-related gene expression, and cell cycle regulation and its possible role in thymineless death and enhanced IR cytotoxicity within these heterozygous p53 cell systems. Recently, a p53 mutant tumor cell line treated with hydroxyurea, 5-FUra, and interferon was reported to have similar dNTP perturbations and S-phase arrest, resulting in cell death (62).

In conclusion, the biochemical and cellular responses of these TS-mutant human colon cancer cells are consistent with our proposed model of FP-RS. We suggest that RS appears to be dependent on progression into S-phase under conditions of an increased dATP:dTTP ratio, resulting in inhibition of DNA synthesis/repair, and the generation of DNA fragmentation and enhanced cell death. A better understanding of this mechanism may be useful in the development of more effective FP-RS regimens for clinical cancer therapy. Further evidence in support of this hypothesis may well come from studies of regulated gene expression after IR.

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