Ratio of 2'-Deoxyadenosine-5'-triphosphate/Thymidine-5'-triphosphate Influences the Commitment of Human Colon Carcinoma Cells to Thymineless Death

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ABSTRACT

In colon cancers induction of a thymineless state following inhibition of thymidylate synthase (TS) by 5-fluorouracil combined with leucovorin can initiate a cytotoxic response. Using a 5-fluorouracil-leucovorin-treated human colon carcinoma cell line (GC_c1) and a clonally derived TS^- mutant, initiation events that dictate the onset of and commitment to thymineless death have been examined. Initial events related to a temporarily associated decrease in dTTP and elevation in the dATP pool; no depletion of dGTP or elevation in dCTP was detected. Nucleosomal degradation of DNA commenced at 24 h in TS^- and 49 h in GC_c1, and was associated with the more rapid development of an imbalance in the dATP and dTTP pools and a higher dATP:dTTP ratio in TS^- cells. The contribution of elevated dATP or depleted dTTP pools to thymineless death was subsequently determined by treatment of GC_c1 or TS^- cells with deoxyadenosine to elevate the dATP pool either under thymidine-replete or thymineless conditions. Thus, deoxyadenosine supplementation under dTTP-replete conditions elevated the dATP pool for 16 h and was cytotoxic to cells. During dTTP depletion elevated dATP was maintained, and cytotoxicity was significantly and rapidly enhanced by deoxyadenosine but could be reversed by thymidine. Data suggest that maintenance of elevated dATP and the dATP:dTTP ratio are essential initiation events in the commitment of colon carcinoma cells to thymineless death.

INTRODUCTION

Thymineless death, originally described in bacteria under conditions of thymine deficiency (1), occurs in mammalian cells following the inhibition of TS^ function. In the absence of dThd salvage capability, inhibition of the synthesis of the only de novo source of dTMP, and hence of dTTP, results in the commitment of cells to die via the consequences of dTTP depletion. Inhibition of TS leads to a decrease in DNA synthesis followed by a period of unbalanced growth where the synthesis of RNA and protein continue (1). Depletion of dTTP has been induced by treatment of both human (2–4) and rodent (5–7) cell lines with agents (FUr or FdUrd) that inhibit TS activity following intracellular metabolism. Alternatively, two groups have derived genetically marked mutants of TS-deficient cells that commit to thymineless death on dThd deprivation (8–11). Only one of these TS^- cell lines has been isolated from human cells (10, 11).

Thymineless death clearly requires DNA replication to occur and is associated with fragmentation of DNA (12). However, the mechanism of induction of thymineless death has remained ill-defined, and may vary dependent upon cell type (2–18). Upon inhibition of TS function, the accumulation of the natural substrate for the TS reaction, dUMP, and subsequently dUTP, or in the case of FUr or FdUrd, dUMP and subsequently dFdUTP, may occur. In a dTTP-depleted state, this may lead to a futile round of incorporation of fraudulent base(s) into DNA followed by excision by uracil-DNA glycosylase that may contribute to the process of DNA strand breakage (12, 19). In some cell lines, the accumulation of dUTP (12, 14, 17, 18) or dFdUTP (15, 16) and incorporation into DNA may be associated with toxicity, whereas in others the lack of accumulation has indicated that a correlation does not exist (8, 14). Additionally, the changes in levels of dATP, dGTP, and dCTP during dTTP depletion that could be important initiation events in the onset of thymineless death have varied considerably among different cell lines (4–7, 14), and may influence the effects on DNA synthesis, the duration of onset or extent of the resulting DNA damage, and cytotoxic response.

In human colon carcinomas, induction of a thymineless state is an important mechanism of cytotoxicity. In cultured cells, FUr-LV cytotoxicity is reversible by dThd (20), and in human colon adenocarcinoma xenografts that lack dThd salvage capability, FUr-LV has demonstrated therapeutic activity (21). This has been substantiated in randomized Phase III clinical trials in patients in whom the activity of FUr alone has been compared with FUr combined with LV, which targets the FUr metabolite FdUMP to the TS locus. Thus, response rates to FUr have ranged from 7 to 15% and to FUr-LV from 33 to 48% (22–25). Using a FUr-LV-treated human colon carcinoma cell line, or a clonally derived TS^- mutant that commits to cell death upon dThd deprivation, we have determined the changes in deoxyribonucleoside triphosphate pools that occur early in the course of cell commitment to thymineless death. Potentially these events are responsible for the induction of a cytotoxic response. In support of such a hypothesis, we report that DNA...
damage and cytotoxicity appear to correlate with increased dATP, which requires a depleted dTTP pool to remain elevated. Supplementation with dAdo that significantly elevates the pool of dATP and induces cytotoxicity under dTTP replete conditions significantly potentiates cellular cytotoxicity under conditions of dTTP depletion. Data suggest that an imbalance in the dATP and dTTP pools, and the size of the dATP:dTTP ratio are significant events in the initiation and onset of thymineless death in colon cancer cells.

MATERIALS AND METHODS

Cell Lines. The cloned human colon adenocarcinoma cell line GC3/c1 (doubling time 24 h) has been described previously (20). The TS° mutant of GC3/c1 (GC3/c1TS°c3/c3), deficient in TS mRNA and protein and auxotrophic for dThd, has been described previously (11). The TS mutant of GC3/c1 (GC3/c1TSc3/c3), deficient in TS mRNA and protein and auxotrophic for dThd, has been described previously (10, 11). For all studies, cell lines were routinely maintained in folate-free RPMI 1640 medium (Hydclone, Logan, UT) containing 10% dialyzed fetal bovine serum (Sigma Chemical Co., St. Louis, MO), physiological folate (80 nM [6RS]5-methyltetrahydrofolate), 712 μM Ca++, and in the case of TS°, 20 μM dThd.

Clonogenic Assays. Cells were plated at a density of 3000 (GC3/c1) or 6000 (TS°) cells/well in Falcon 6-well plates (Becton Dickinson Labware, Lincoln Park, NJ), and allowed to attach overnight. TS° and GC3/c1 cells subsequently received either treatment with dAdo (1–3 mM) for periods of up to 24 h, either in the presence of dThd (20 μM) or following dThd deprivation (TS°; Ref. 11) or FUra (1 μM)-LV (1 μM) treatment (GC3/c1; Ref. 20) to initiate a thymineless state. Alternatively, the influence of dThd supplementation or dTTP depletion alone was also examined for periods up to 96 h. At the end of drug treatment for the designated times, cells were refed in the presence of dThd (20 μM), and clonogenic survival was determined after an additional 7 days (GC3/c1) or 11 days (TS°). Plates were prepared and colonies enumerated as described previously (11, 20).

HPLC Analysis of dNTP Pools. TS° and GC3/c1 were seeded in T-162 flasks (Costar, Cambridge, MA) at a density of 5 × 10⁶ cells and allowed to enter log phase growth (2–3 days). Following the various treatment conditions described for clonogenic assays, 20 × 10⁶–60 × 10⁶ cells/flask were harvested for periods of up to 48 h following the initiation of treatment. Monolayers were initially washed in 10 ml HBSS, and harvested using 10 ml trypsin (0.05%)-EDTA (0.53 mM) for 3 to 4 min, 37°C, followed by 10 ml ice-cold RPMI 1640 medium containing 10% diazoyed fetal bovine serum. Cells were resuspended under the same conditions, counted, and subsequently washed twice in ice-cold PBS containing 0.1% glucose. Nucleotides were extracted in perchloric acid followed by neutralization with freon/tri-n-octylamine according to the method of Pogolotti et al. (26). Removal of ribonucleotides from the aqueous layer was accomplished by periodate oxidation, as previously described (27). The extraction efficiency obtained for dNTPs was 85%. Deoxyribonucleoside triphosphates were resolved by HPLC analysis using two separate procedures: (a) dCTP, dATP, dGTP, dUTP, and dTTP were separated by ion-pair reverse-phase HPLC methodology using an Alltech 7-μm nucleotide/nucleoside column (250 x 4.6 mm), with RTs of 6.0, 22.3, 27.9, 34.5, 41.4, and 43.1 min, respectively. Buffers were: A, 0.2 M (NH₄)₂PO₄-5 mM tetraethylammonium hydrogen sulfate (pH 3.0) and B, Buffer A + 30% methanol. The flow rate was 1.5 ml/min. Separation was effected using 100% Buffer A isocratically for 15 min followed by a gradient to 30% Buffer B for 25 min; at 50 min, the gradient was increased to 80% Buffer B over 5 min. (b) A strong anion exchange procedure using a Whatman Partisil 10 SAX column (250 x 4.6 mm), as described (28). The RTs for dCTP, dTTP, dUTP, dATP, and dGTP were 11.8, 15.2, 15.9, 16.2, 17.7, and 27.1 min, respectively. HPLC analysis was conducted using a Beckman Gold Star 126 HPLC system fitted with a 166 detector and 507 autosampler. Data were integrated and compiled using a dedicated IBM Model 50 PC. The authenticity of dNTPs was determined from the RTs of known standards, and the A₂₆₀/A₂₃₀, UV absorbance ratios of the unknown peaks. The limit of detectability for dNTPs was 1 pmol/10⁶ cells (from an injection volume equivalent to 5 × 10⁶ cells).

Nucleosomal Ladder Formation. The onset of nucleosomal ladder formation was examined for up to 72 h after dThd deprivation (TS°) or FUra (1 μM)-LV (1 μM) treatment (GC3/c1). Cells were plated, harvested, and DNA was extracted in phenol-chloroform and analyzed by electrophoresis in 1.2% agarose gels as described previously (11).

RESULTS

Relationship between Thymineless Stress and Clonogenic Survival. Asynchronously growing TS° cells (deficient in TS) were deprived of dThd to induce a thymineless state. Alternatively, parental GC3/c1 cells with functional TS activity were treated with FUra (1 μM)-LV (1 μM) that targeted FUra to the TS locus (20). In TS° cells, the loss in clonogenic survival occurred earlier than in GC3/c1 (Table 1). The time required for a 50% decrease in survival was approximately 55 h in TS° and 62 h in GC3/c1.

Influence of Thymineless State on dNTP Pools. Following dThd withdrawal from TS° cells or FUra-LV treatment of GC3/c1 cells, dNTP pools were determined for up to 48 h. At this time, 27–40% of the cells had lost clonogenic potential if dThd or drug-free medium was restored. These data indicate that the commitment of cells to thymineless death occurred during
this period. In TS−, dGTP pools remained low at 3 pmol/10^6 cells for the duration of the experiment. In contrast, the dCTP pool decreased rapidly by 2 h, returning to control levels by 16–24 h (Fig. 1). The major prolonged changes in the sizes of dNTP pools were observed to occur in the dTTP and dATP pools. The initial level of dTTP was 8 pmol/10^6 cells, and decreased rapidly upon dThd deprivation, reaching undetectable levels by 16 h. Concomitant with a decline in the dTTP pool was a gradual elevation in dATP from a basal level of 21 pmol/10^6 cells at time 0, to 62 pmol/10^6 cells, 24 h after dThd withdrawal. This was maintained in parallel with a depleted dTTP pool. In none of the samples was dUTP detected.

Similarly, changes in dNTP pools were examined in asynchronously growing GC3/cl cells following exposure to FUra (1 μM)-LV (1 μM), Fig. 2. Of interest were the initial lower levels of dATP and higher dTTP, which were 5 and 13 pmol/10^6 cells, respectively, in comparison to TS− (21 and 8 pmol/10^6 cells, respectively). Upon FUra-LV treatment, a more gradual decrease in dTTP was observed, reaching a nadir between 24 and 48 h. Temporally associated with the decline and continued depletion of dTTP was an elevation in dATP by 24 h to 36 pmol/10^6 cells, which was maintained for the 48 h examined, and could be abolished by dThd (20 μM) supplementation (data not shown). Hence, the ratio of dATP:dTTP increased more gradually in GC3/cl than in TS− during the first 24 h after induction of the thymineless state. Minimal fluctuations in the dCTP and dGTP pools were detected. Additionally, neither dUTP nor dFdUTP was detected.

**Nucleosomal Ladder Formation.** As a measure of DNA damage, the onset of nucleosomal ladder formation was examined following dThd withdrawal in TS− cells (Fig. 3) or FUra-LV treatment of GC3/cl cells (Fig. 4). Consistent with the loss of clonogenic survival in TS− at 24 h after dThd deprivation, fragmentation of the DNA was initially detected at this time and increased with further exposure to thymineless conditions, being clearly evident at 32 h. In FUra-LV-treated GC3/cl cells, the detection of internucleosomal DNA fragments occurred later, with fragmentation of the DNA being readily detectable at 49 h.

**Influence of the dATP and dTTP Pools on Commitment to Thymineless Death.** The relationship between the dATP pool, dTTP pool, and cytotoxicity was examined further by using dAdo and/or dThd supplementation in TS− and GC3/cl cells to manipulate the size of the two pools.

In TS− cells, following supplementation with 20 μM dThd and 1 mM dAdo (Fig. 5A), the dTTP pool was maintained at normal levels for the duration of the experiment (48 h), whereas the dATP pool rose rapidly, reaching a maximum of 101 pmol/10^6 cells at 4 h followed by a decline to control levels by 16 h and a more gradual decline over the next 32 h. As a consequence of the elevation of dATP, the dCTP pool was transiently depleted in temporal association with elevated dATP, and recovered to control levels by 16 h to 10 pmol/10^6 cells; no change in the dGTP pool was detected. When dAdo (1 mM) was added concomitantly with dThd withdrawal (Fig. 5B), similar changes occurred in the dGTP and dCTP pools. dTTP was completely undetectable from 8 h onward under these conditions. Of interest was that the dATP pool increased in a manner identical to that in the presence of dThd; however, dATP did not continue to decline beyond 16 h under conditions of dTTP depletion, but remained elevated and continued to further increase, reaching a level of 76 pmol/10^6 cells at 48 h. These data for dATP between 16 h and 48 h paralleled the rate of increase of dATP observed in the absence of dThd or dAdo supplementation, suggesting that depletion of dTTP was necessary to maintain an elevated dATP pool.
Similarly, in GC/c1 cells, supplementation with dAdo (3 mm) induced a rapid elevation in the dATP pool, which was similar under dTTP-depleted or dTTP-replete conditions for the first 24 h. A comparison of GC/c1 cells treated with FUra-LV alone (Fig. 2) or FUra-LV in the presence of 3 mm dAdo (Fig. 6) indicated temporally similar changes in the dNTP pools to those observed in TS cells. Thus, dATP was rapidly elevated within 2 h to 24 pmol/10^6 cells and was associated with a more rapid decline in the dTTP pool and a transient decrease in dCTP. However, beyond 24 h, the increase in dATP paralleled the maintenance of a depleted dTTP pool.

**Potentiation of Cytotoxicity by dAdo.** Since the duration of exposure to elevated dATP could be enhanced in TS and GC/c1 cells by treatment with dAdo, and the dATP:dTTP ratio could be increased after induction of the thymineless state or reduced by dThd supplementation, the influence of dAdo on clonogenic survival was determined during drug exposures of up to 24 h (Fig. 7). In GC/c1, a 10% loss in clonogenic survival was detected following a 4-h exposure to 3 mm dAdo alone, increasing to 70% at 24 h, and was independent of the presence of dThd. Using FUra (1 μM)-LV (1 μM) (that did not initiate a cytotoxic response during the initial 24 h of drug exposure in the absence of dThd), cytotoxicity was determined in the presence of 3 mm dAdo. The combination was significantly more toxic than dAdo alone, causing 88% loss in clonogenicity within 8 h. FUra-LV-dAdo cytotoxicity was reduced by dThd (20 μM) to levels observed with dAdo treatment alone. Similarly, treatment of TS cells with 1 mm dAdo in the presence of dThd was cytotoxic, with a 35% loss in clonogenicity by 4 h; dAdo cytotoxicity was also potentiated in the thymineless state, elevating the level of cell kill to 50% and 70% at 4 and 8 h, respectively.

**Fig. 3** Computer-enhanced image of the onset of nucleosomal ladder formation in asynchronously growing TS cells both prior to (time 0) and following dThd withdrawal for up to 72 h. DNA was extracted in phenol-chloroform, and the formation of nucleosomes was examined by agarose gel electrophoresis as described in “Materials and Methods.”

**Fig. 4** Computer-enhanced image of the formation of nucleosomal DNA following treatment of GC/c1 cells continuously with FUra (1 μM)-LV (1 μM) for periods up to 72 h, and analysis in 1.2% agarose gels as described in “Materials and Methods.”

**DISCUSSION**

Several studies have reported perturbations in dNTP pools, in particular in rodent cell lines, following treatment with FUra or FdUrd (4–7). The multiplicity of changes detected, along with a lack of assessment of the potential role of dUTP or FdUTP in the onset of thymineless death, have rendered it difficult to evaluate whether certain signaling events were causative or merely associated with the commitment to cell death. In mouse L5178Y cells (5), FUra treatment induced rapid depletion of dTTP and rapid elevation of dCTP to 280% of control. A more gradual rise in dGTP and dATP to 140 and 250% of control, respectively, occurred. These data suggested that the kinetics of dTTP depletion and dCTP elevation was temporally associated. In preclinical model systems of FdUrd-treated human neuroblastomas in nude mice or FdUrd-treated rat embryos, elevation of dCTP and depletion of dTTP were also demonstrated, while dATP was modestly increased by 38–64%. However, toxicity appeared to correlate temporally with a precipitous decline in dGTP (4). Similarly, in FdUrd-treated mouse FM3A cells and dThd-deprived TS variants, a rapid decline in dTTP and associated depletion of dGTP were detected, with a 70% elevation in dATP and minimal change in the dCTP pool (7). In mouse FM3A cells, dAdo supplementation at a concentration of 3 mm elevated the dATP pool by 100-fold at 6 h; the dGTP pool was also rapidly depleted, and a 90% reduction in dCTP occurred at 10 h (29). This study led to the conclusion that the imbalance between the dATP and dGTP pools may be the trigger for cell death in FdUrd-treated cells. However, dAdo supplementation was at a concentration of 19-fold above the EC_{50}, and a 100-fold elevation in the dATP pool, which was not obtained in FdUrd-treated cells, may not be physiologically relevant.

It was evident from preliminary studies conducted in the GC/c1 and TS colon carcinoma cell lines that very characteristic patterns of perturbation in dNTP pools were demonstrated following induction of the thymineless state that were different from those observed in rodent models. Specifically, the dGTP pool was not depleted nor was dCTP elevated. In addition, both
Fig. 5  Influence of dAdo (1 mM) treatment on dNTP pools was examined in TS cells in the presence of dThd (20 μM) or during 48 h after dThd withdrawal. Data represent mean ± SD of two to four determinations at each time point. A, +dAdo + dThd; B, +dAdo − dThd. ■, dATP; ▲, dTTP; ○, dCTP; ▼, dGTP.

Fig. 6  To examine the influence of dAdo treatment on dNTP pools in GC3/c1 under thymineless conditions, cells were treated with FUra (1 μM)-LV (1 μM) in the presence of dAdo (3 mM) for up to 48 h. Each time point, mean ± SD of two to four determinations. ■, dATP; ▲, dTTP; ○, dCTP; ▼, dGTP.

The mouse FM3A cell line and its derived TS− mutant demonstrated considerably different kinetics of thymineless death in comparison to GC3/c1 or TS− (7, 8). Following initiation of thymineless stress in the mouse FM3A models, DNA strand breaks and a 50% loss in clonogenic survival occurred within 6 h, presumably related to a shorter doubling time (12 h). In addition, a precipitous decline in dGTP and a modest increase in dATP were detected. In GC3/c1 and TS−, however, the onset of thymineless death, as determined by nucleosomal ladder formation and loss in clonogenic survival, correlated with a temporally associated decrease in dTTP and rise in the dATP pool. In cell lines where dUTP or FdUTP have been considered to play a significant role in the mechanism of induction of thymineless death, these dNTPs, in particular dUTP, have accumulated to relatively high levels within cells (90–338 pmol/10⁶ cells; Refs. 14, 17, 18, 30–32). However, in TS− and GC3/c1, neither dUTP nor FdUTP were detected.

The onset of thymineless death has correlated with the formation of single- and double-strand breaks in DNA and DNA fragmentation, requiring a period of time for the accumulation of DNA damage with loss of viability (7, 8, 13, 18, 30–33). Consistent with these observations, the formation of nucleosomal ladders in DNA was readily detected at 49 h in GC3/c1 cells, and correlated with the appearance of significant DNA strand breakage at 48 h as determined by alkaline elution (30). The expression of DNA damage appeared suddenly as reported for FdUrd-treated mouse FM3A cells (7), and correlated with loss in clonogenic survival. These findings are consistent with late events in the induction of DNA damage (30). The onset of nucleosomal ladder formation occurred earlier in TS− than in GC3/c1, and was paralleled by the more rapid development of an imbalance in the dATP:dTTP pools following induction of the thymineless state. It is evident that prior to the detection of DNA fragmentation that correlated with activation of the cell death program, both GC3/c1 and TS− cells could be rescued with dThd. Thus, breaks in high molecular weight DNA that may be initial events in the initiation of DNA damage following perturbation of dNTP pools by fluoropyrimidines (33) are presumably dThd reversible, although these were not determined in the present study.
A potential consequence of initiation of the thymineless state might be to arrest cells at the G1-S boundary, thereby preventing entry into S-phase. However, TS cells released from G0 synchrony in the absence of dThd appeared to enter the S-phase 12–14 h after release (11), and subsequently lost clonogenic potential. In addition, aphidicolin, an inhibitor of DNA polymerase α that is frequently used to synchronize cells at the G1-S interface, failed to induce DNA fragmentation or cytotoxicity in two human colorectal tumor cell lines in contrast to treatment with FdUrd (13). Taken together, these data suggest that during dTTP depletion colon carcinoma cells may continue to enter the S-phase and initiate DNA replication.

Perturbations in dNTP pools are regulated at the level of ribonucleotide reductase (34–36). Although the allosteric regulation of this enzyme is complex, reduction of ADP to dADP is activated by low concentrations of dTTP, thereby generating elevated levels of dATP (34). In addition, with the onset of DNA damage, the expression of this enzyme may be induced to facilitate DNA repair processes (37), and may lead to even higher intracellular concentrations of dATP. Another factor that may contribute to the maintenance of an elevated dATP pool in GC/c1 and TS cells is the expression of only very low levels of adenosine deaminase.4 This enzyme is responsible for the deamination of both adenosine and dAdo, and enzyme-deficient patients suffering from severe combined immune deficiency lack functional T and B lymphocytes as a consequence of elevated cellular dATP (38).

In both TS and GC/c1, the temporal association between depleted dTTP, elevated dATP, and the onset of cell death suggested that the initiation event in the commitment of colon carcinoma cells to thymineless death may be due to the extent of the imbalance between the two pools. Thus, supplementation of TS or FURA-LV-treated GC/c1 cells with dAdo under dThd replete conditions should be cytotoxic due to elevation of the dATP:dTTP ratio, and in the thymineless state should increase the cytotoxic response due to a further imbalance in the dATP:dTTP ratio.

The importance of dATP in contributing to the initiation of a cytotoxic response in colon carcinoma cells was evident when dAdo treatment alone was found to be cytotoxic under dTTP-replete conditions. However, in GC/c1 cells under conditions of dTTP depletion, cytotoxicity in the presence of dAdo was significantly enhanced, whereas in the presence of dThd, the toxicity of FURA-LV-dAdo was identical to that caused by dAdo alone. These events may be similar to the dATP-induced lymphotoxicity described in adenosine deaminase-deficiency states (38), or following the treatment of lymphocytes with dAdo (39). Of importance was the finding that, in the presence of dTTP, dAdo could elevate dATP transiently, and a 16-h exposure was clearly sufficient to induce a cytotoxic response. However, in the dTTP-depleted state, where higher ratios of dATP:dTTP were generated rapidly in the presence of dAdo, cytotoxicity was also rapidly enhanced and could be consistent with the exacerbation of breaks in high molecular weight DNA. These data therefore suggest that the ratio of dATP:dTTP and duration of maintenance of the imbalance are important determinants of the onset and commitment of colon carcinoma cells to thymineless death. Since these colon carcinoma cells are low in adenosine deaminase activity and sensitive to elevated dATP pools in particular during dTTP depletion, it may be feasible to explore the possibilities for combining FURA-LV with dAdo. This could be conducted in the presence of the adenosine deaminase inhibitor deoxycoformycin to prevent degradation of

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4 Unpublished observations.
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