A77 1726 Induces Differentiation of Human Myeloid Leukemia K562 Cells by Depletion of Intracellular CTP Pools

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ABSTRACT

A77 1726 (LEF) is the active metabolite of leflunomide, a recently approved immunosuppressive agent. We examined the ability of LEF to induce differentiation of a human erythroleukemia (K562) cell line and show that LEF induces a dose- and time-dependent differentiation of these cells as characterized by growth inhibition, hemoglobin production, and erythroid membrane protein glycophorin A expression. This effect was dependent on depletion of the intracellular pyrimidine ribonucleotides (UTP and CTP), and preceded by a specific S-phase arrest of the cell cycle. Supplementation of the cultures with exogenous uridine restored intracellular UTP and CTP to normal levels and prevented the LEF-induced cell cycle block and differentiation of K562 cells. Interestingly, addition of cytidine alone blocked the LEF-induced differentiation of K562 cells but only restored the CTP pool. By contrast, neither deoxyctydine nor thymidine prevented the effects of LEF on these cells. Similarly, pyrimidine starvation of a cell line lacking the de novo pyrimidine pathway (G9c) resulted in an S-phase arrest that was reversed by the addition of cytidine. Thus these studies demonstrate an important role for CTP in regulating cell cycle progression and show that LEF is an effective inducer of tumor cell differentiation through depletion of this ribonucleotide.

Rheumatoid arthritis (RA) is a complex autoimmune disease characterized by deregulated proliferation of lymphocytes, chronic inflammation and progressive erosion of cartilage and bone (Herrmann et al., 2000). A recently approved agent for the treatment of RA is leflunomide (Arava; N-[4-(trifluoromethyl)-phenyl] 5-methylisoxazole-4-carboxamide), a novel immunomodulatory drug that has demonstrated immunosuppressive activity in animal model studies of transplantation, lupus, and myasthenia gravis (Fox et al., 1999; Herrmann et al., 2000; Pirschewer et al., 2001). Upon first-pass metabolism, leflunomide is rapidly converted to the pharmacologically active metabolite A77 1726 (abbreviated as LEF in this report). Although the mechanism of LEF action has been controversial, considerable evidence now suggests that the immunosuppressive effects of LEF are mediated through the inhibition of dihydroorotate dehydrogenase, the fourth enzyme in the de novo pyrimidine nucleotide synthesis pathway (Cherwinski et al., 1995; Nair et al., 1995; Williamson et al., 1995; Zielinski et al., 1995; Xu et al., 1996; Fox et al., 1999).

Contributing to the controversy, both unmetabolized leflunomide (also known as SU101) and LEF show inhibitory activity against tyrosine kinases (Ghosh et al., 1998; Mahajan et al., 1999). SU101 and LEF have been demonstrated to inhibit signal transduction mediated by platelet-derived growth factor or epidermal growth factor receptor (Xu et al., 1996, 1999; Elder et al., 1997; Ghosh et al., 1998; Strawn et al., 2000) and the phosphorylation of Jak1 and 3, which are necessary for interleukin-2 receptor signaling (Elder et al., 1997). LEF-A12, LEF-A13, and other analogs of LEF also show antitumor potential through inhibition of the epidermal growth factor receptor tyrosine kinase or Bruton’s tyrosine kinase (BTK) (Ghosh et al., 1998; Mahajan et al., 1999). However, the concentrations of LEF required for inhibition of tyrosine kinases are considerably higher than the IC50 values for growth inhibition of various mammalian cells and the effects of LEF on the murine leukemia cell line LSTRA (IC50 = 10–30 μM) were reversed by the addition of exogenous uridine, suggesting that the antiproliferative activity of LEF on these cells occurred through inhibition of de novo pyrimidine nucleotide synthesis (Xu et al., 1996).

The proliferation of T lymphocytes is highly dependent on pyrimidine availability; in response to mitogens T lymphocytes expand their pyrimidine pools approximately 8- to 10-
fold (Fairbanks et al., 1995; Ruckemann et al., 1998) and interruption of pyrimidine synthesis inhibits the proliferation of these cells (Ruckemann et al., 1998). In mammalian cells, pyrimidine ribonucleotides are synthesized by two major routes, either through the de novo synthesis from glutamine, ATP, and bicarbonate (Jones, 1980) or salvage pathway synthesis from uridine or cytidine (Traut, 1994). Uridine/cytidine kinase is the rate-limiting enzyme in the salvage pathway and provides a potential mechanism to prevent pyrimidine limitation in response to inhibition of de novo synthesis. Although most mammalian cells have the capacity to synthesize pyrimidine ribonucleotides by either route, the dependence on the de novo or salvage pathway varies considerably with cell type (Traut, 1994).

In tumor cells, there is substantial evidence for increased rates of nucleotide synthesis. The activities of rate-limiting enzymes such as CAD, CTP synthetase, thymidylate synthase, dihydrofolate reductase, IMP dehydrogenase, ribonucleotide reductase, and uridine/cytidine kinase are significantly increased through changes in expression, phosphorylation, or other mechanisms of regulation (reviewed in Hatse et al., 1999a). Consequently, tumor cells show substantial increases over normal cells in both deoxy ribonucleotides (6- to 11-fold) and ribonucleotides (1.2- to 5-fold) (Traut, 1994). Thus, a current strategy of cancer pharmacology is to disrupt the balance among intracellular (deoxy) ribonucleotide pools through targeted inhibition of nucleotide biosynthetic enzymes and thereby induce differentiation (reviewed in Hatse et al., 1999a) and/or apoptosis of tumor cells (James et al., 1997).

Despite the promising use of LEF for autoimmune disorders, the application of LEF for the treatment of leukemias has only recently been considered. The human K562 cell line was isolated and characterized from a patient with chronic myelogenous leukemia in blast crisis (Lozzio and Lozzio, 1975) and is widely used as a model system for the study of cell differentiation. These cells exhibit a low proportion of hemoglobin-synthesizing cells under standard cell growth conditions but are capable of undergoing erythroid differentiation (reviewed in Hatse et al., 1999a) and/or apoptosis of tumor cells (James et al., 1997).

Using erythroid differentiation as a surrogate marker for growth inhibition, we evaluated the effects of LEF and show that this compound depletes intracellular pyrimidines and induces the differentiation of these cells, independently of effects on tyrosine phosphorylation. Concordant with these effects, we observed an S-phase arrest that was prevented by restoration of CTP pools; these results were recapitulated in a cell line lacking the de novo synthetic pathway. Thus, these studies point to an essential role for CTP in determining cell cycle progression and potentially influencing the balance between cell proliferation and differentiation.

**Materials and Methods**

**Cell Culture and Reagents.** Human erythroleukemia K562 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were kept at 37°C in an atmosphere containing 5% CO₂. The stock solution of LEF was prepared as a 250 mM dimethyl sulfoxide solution and stored at −20°C. Control cells were treated with equivalent amounts of dimethyl sulfoxide; benzidine, propidium iodide, thymidine, cytidine, and uridine were from Sigma-Aldrich (St. Louis, MO). Anti-glycerophorin A phycoerythrin (PE) was obtained from BD Biosciences, San Jose, CA. [γ-<sup>32</sup>P]ATP (3000 Ci/mmol) was obtained from PerkinElmer Life Science (Boston, MA).

**Cell Cycle Analysis.** Cells (5 × 10⁶) were fixed in 1 ml of cold 70% ethanol for at least 12 h at 4°C or −20°C until analysis. Cells were spun down, rinsed once with PBS, and resuspended in 0.5 ml of PBS. After incubation in 50 μg/ml RNase A solution for 1 h at 37°C, propidium iodide was added to stain the cells at a final concentration of 50 μg/ml, the cells were mixed well and let stand for additional 1 h at 4°C. DNA fluorescence was measured by fluorescence-activated cell scanning using a FACSScan flow cytometer (BD Biosciences, San Jose, CA) and percentage of cells within the G₁, S, and G₂/M phases of the cell cycle were determined by the ModFit cell-cycle analysis program (Verity Software, Topsham, ME).

**Measurement of Erythroid Differentiation of K562 Cells by Benzidine Staining.** Erythroid differentiation was determined by measuring hemoglobin production by benzidine staining (Nagy et al., 1995). Benzidine dihydrochloride (2 mg/ml) was prepared in 0.5 M (3%) acetic acid, and H₂O₂ (1%) was added immediately before use. The cell suspensions were mixed with the benzidine solution in a 1:1 ratio and counted in a hemocytometer after 5 min. Blue cells were considered positive for hemoglobin and at least 1000 cells were counted per sample.

**Analysis of Intracellular Ribonucleotides by HPLC.** K562 cells (1.0 × 10⁶) treated in various conditions were harvested by centrifugation (2000 rpm, 5 min) and washed twice with ice-cold PBS. The cell pellets were suspended in 1 ml of ice-cold 10% trichloroacetic acid and briefly vortexed. The precipitated protein was removed immediately by centrifugation (2 min at 10,000 rpm) and the supernatant was then extracted four to five times with water-saturated diethyl ether until the pH was above 5.0. A portion (500 μl) of the extract was reduced to a volume of approximately 200 to 250 μl by SpeedVac (Thermo Savant, Holbrook, NY). HPLC analyses were performed as described previously (Pogolotti and Santi, 1982). A portion (100 μl) of the filtered sample was injected onto a SAX Partisil 5X HPLC column (Whatman, Clifton, NJ) at a flow rate of 1 ml/min. The running buffer (buffer A) was composed of 7 mM NH₄H₂PO₄, pH 3.8, and the elution buffer (buffer B) contained 250 mM NH₄H₂PO₄, pH 4.5, and 500 mM KCl. After 6 min of an isocratic period with buffer A, a linear gradient of buffer B was applied for 30 min followed by an additional isocratic period of 10 min of buffer B. Ribonucleotide standards (ATP, GTP, CTP, and UTP) were also run under the same conditions and were used to quantify the amounts of ribonucleotides obtained from the cell lysates.

**Flow Cytometric Assessment of Glycerophorin A Expression.** Untreated and drug-exposed K562 cells (1 × 10⁶ cells/sample) were collected and washed twice with PBS, then resuspended in 200 μl of PBS. PE (20 μg/ml)-conjugated antibodies against glycerophorin A were added to stain the cells. After incubation at room temperature for 30 min, the stained cells were washed twice in PBS, fixed with 500 μl of ice-cold 1% paraformaldehyde in PBS and stored at 4°C. The fluorescence of the cells was then measured on a FACSScan flow cytometer (BD Biosciences) equipped with CellQuest software (BD Biosciences). Cell debris was excluded from the analysis by conventional gating of forward scatter versus side scatter dot plots.

**Immunoblots.** Attached cells were washed with ice-cold PBS, and then collected in a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 150 μM Na₃VO₄, 0.25 mM phenylmethysulfonyl fluoride, 5 μg/ml leupeptin, and 10 mM microcystin LR. After centrifugation (15,000g, 10 min, 4°C), the protein content in the supernatant was assayed using the method of Bradford (1976) (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Equal amounts of protein were applied to an SDS-polyacrylamide gel electrophoresis and transferred to
polystyrene/silane-coated, 96-well plates) were washed with culture medium containing 2 mM EDTA, 150 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol in culture. Erythroid cell differentiation is characterized by the induction of K562 cell Differentiation by LEF. Human erythroid K562 cells were used as a model cell line to investigate the effects of LEF on leukemia cell differentiation in culture. Erythroid cell differentiation is characterized by increased synthesis of hemoglobin and is readily quantitated by benzidine-positive cell staining. Spontaneous differentiation of K562 cells resulted in approximately a 3%–5% increase in benzidine-positive cells after 5 days whereas treatment with LEF at concentrations from 1.5 to 25 μM increased the percentage of benzidine-positive cells in a dose-dependent manner. After 96 to 120 h of exposure to LEF, the differentiation of these cells reached a plateau level of 50% to 60% (Fig. 1), and at the highest concentration of LEF tested (25 μM), approximately 28% of the cells underwent apoptosis (data not shown). Coincubation of K562 cells with uridine (30 μM), prevented the LEF-dependent increase in benzidine-positive cells, suggesting that LEF was inducing differentiation in a pyrimidine nucleotide-dependent manner (Fig. 1).

Effects of Uridine or Cytidine on LEF-Induced Inhibition of Cell Growth and Differentiation of K562 Cells. To investigate if the LEF-dependent induction of K562 cell differentiation was dependent on depletion of uridine or cytidine ribonucleotides, cells were incubated with uridine or cytidine to increase intracellular UTP and CTP, respectively, through salvage pathway synthesis. K562 cells were incubated with 25 μM LEF for 48, 72, or 96 h in the absence or presence of 25 μM LEF plus 30 μM uridine, 100 μM cytidine, or 100 μM thymidine, respectively, at times indicated. A, values of cell numbers are the means ± S.D. of triplicate samples. B, intracellular hemoglobin was detected by benzidine-staining and positive stained K562 cells were counted under microscopy using 100× magnification.

Results

Induction of K562 cell Differentiation by LEF. Human erythroid K562 cells were used as a model cell line to investigate the effects of LEF on leukemia cell differentiation in culture. Erythroid cell differentiation is characterized by increased synthesis of hemoglobin and is readily quantitated by benzidine-positive cell staining. Spontaneous differentiation of K562 cells resulted in approximately a 3%–5% increase in benzidine-positive cells. Spontaneous differentiation of K562 cells resulted in approximately a 3%–5% increase in benzidine-positive cells after 5 days whereas treatment with LEF at concentrations from 1.5 to 25 μM increased the percentage of benzidine-positive cells in a dose-dependent manner. After 96 to 120 h of exposure to LEF, the differentiation of these cells reached a plateau level of 50% to 60% (Fig. 1), and at the highest concentration of LEF tested (25 μM), approximately 28% of the cells underwent apoptosis (data not shown). Coincubation of K562 cells with uridine (30 μM), prevented the LEF-dependent increase in benzidine-positive cells, suggesting that LEF was inducing differentiation in a pyrimidine nucleotide-dependent manner (Fig. 1).

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presence of 30 μM uridine or 100 μM cytidine. The LEF-induced inhibition of K562 cell growth (Fig. 2A) paralleled the induction of differentiation (Fig. 2B) and both effects were completely suppressed by the addition of either uridine or cytidine to the growth media. Interestingly, supplementation with cytidine alone prevented the effects of LEF (Fig. 2). By contrast, thymidine addition (100 μM) was unable to inhibit the effects of LEF and the addition of cytidine and thymidine together was equivalent to that of cytidine alone (Fig. 2), indicating that repletion of the intracellular CTP pool was essential for the resumption of cell growth.

**Prevention of Glycophorin A Expression by Uridine or Cytidine Addition.** To further evaluate the effects of LEF on K562 cell differentiation, the expression of an erythroid-specific marker, membrane antigen glycoprotein A was determined by flow cytometry. Treatment of K562 cells with 12.5 μM LEF for 96 h markedly increased the expression of this protein as demonstrated by a shift in the flow cytometric profile (Fig. 3). Incubation with 30 μM uridine and 100 μM cytidine alone did not effect the expression of glycophorin A whereas the LEF-dependent induction of glycophorin A was completely prevented by incubation with either uridine or cytidine (Fig. 3). Thus, using glycophorin A synthesis as a marker of differentiation further demonstrated that LEF induced cell differentiation in a pyrimidine-dependent manner.

**LEF Treatment Selectively Depletes Cellular Pyrimidines; Restoration of UTP or CTP Pools with Uridine or Cytidine Addition.** To examine the effects of LEF on intracellular pyrimidine nucleotides, soluble ribonucleotide triphosphates were extracted and measured on a Partisil SAX anion exchange column as described under Materials and Methods. After incubation of K562 cells with LEF (6 h), the cellular UTP and CTP levels decreased to approximately 42 and 38% of the control values, respectively; after 24 h, these levels further decreased to 30 and 31% of control, respectively (Fig. 4A). By contrast, the amounts of intracellular ATP and GTP increased initially and then declined, demonstrating that LEF specifically reduced intracellular pyrimidine pools as reported earlier (Fox et al., 1999).

K562 cells were treated with LEF (25 μM, 24 h) and the ability of uridine (30 μM) or cytidine (100 μM) to restore the UTP and CTP pools was determined. Exposure to LEF resulted in the reduction of both UTP and CTP levels to 16 and 18% of control, respectively; whereas, the addition of uridine or cytidine individually restored UTP and CTP levels to approximately 70% of control values (Fig. 4A). The addition of uridine and cytidine together was equivalent to that of cytidine alone (Fig. 4A). The results indicated that LEF caused a selective depletion of intracellular pyrimidine nucleotides and that cellular pyrimidine pools could be restored by the addition of uridine or cytidine. The specific depletion and restoration of UTP and CTP pools by uridine and cytidine, respectively, further demonstrated that LEF specifically affected pyrimidine metabolism.

**Fig. 3.** Effect of uridine and cytidine on glycophorin A expression in K562 cells. After a 4-day exposure to LEF (12.5 μM), in the presence or absence of 30 μM uridine or 100 μM cytidine, K562 cells were stained with PE-conjugated monoclonal antibody against glycophorin A. The fluorescence of the stained cells was measured by flow cytometry as described under Materials and Methods.
LEF plus 30
H9262
represented approximately 37, 56, and 0.7%, respectively, of
H9262
were exposed to 25
H9262
of exogenous uridine and cytidine on cellular nucleotide levels. A, cells
Depletion of cellular pyrimidine pools by LEF exposure and effects
Fig. 4.
S-phase Cell Cycle Arrest That Precedes the Differentiation of K562 Cells. Because our results demonstrated that LEF was inducing K562 cell differentiation, we examined the effect of this compound on cell cycle progression. Flow cytometric analysis demonstrated that in untreated control cell cultures, G1, S, and G2–M–phase cells represented approximately 37, 56, and 0.7%, respectively, of

LEF Induces a Pyrimidine Ribonucleotide-Dependent, S-Phase Cell Cycle Arrest That Precedes the Differentiation of K562 Cells. Because our results demonstrated that LEF was inducing K562 cell differentiation, we examined the effect of this compound on cell cycle progression. Flow cytometric analysis demonstrated that in untreated control cell cultures, G1, S, and G2–M–phase cells represented approximately 37, 56, and 0.7%, respectively, of

the total cell population. After treatment of K562 cells with LEF for 12 h, the cell cycle distribution remained comparable with that of control cells (data not shown). Increasing the time of exposure to LEF increased the percentage of cells in S phase (24 h, 66%; 48 h, 91%) and, after 48h, the S-phase–arrested K562 cells were unable to proceed into the G2–M phase (Fig. 5). Removal of LEF (24 h) from K562 cells resulted in abrogation of the cell cycle arrest, demonstrating that the LEF-induced arrest was reversible (data not shown). Coincubation of LEF-treated cells with uridine or cytidine almost completely blocked the LEF-induced cell cycle arrest, whereas in the absence of LEF, neither uridine nor cytidine affected the cell cycle distribution (Fig. 5; data not shown). Again, the addition of cytidine alone was as effective as uridine at preventing the LEF-induced cell cycle block demonstrating a specific requirement for CTP in this process (Fig. 4B and 5).

Effect of LEF on the Expression of Cell Cycle-Regulatory Proteins in K562 Cells. Several key regulators of S phase initiation, processing, and termination were examined after LEF treatment. The expression of Cdk-2, cyclin A, cyclin E, and p21 were examined by immunoblotting from LEF-treated cells. Although no significant changes in protein expression were found for Cdk2 in LEF-treated K562 cells compared with controls (Fig. 6A), we observed a slight decline in Cdk2 activity at 6 h followed by a consistent increase in activity after 72 h of LEF exposure (Fig. 6B). Consistent with the observed changes in Cdk2 activity, the expression of p21 was first induced after LEF treatment for 6 to 12 h, then reduced after 24-h LEF treatment (Fig. 6A). Concordant with the LEF-induced S-phase arrest, an accumulation of cyclin E and A was observed in LEF-exposed K562 cells and these proteins remained elevated after 72 h in comparison with untreated K562 cells (Fig. 6A). No induction of p53 was detected in response to LEF exposure (25 μM) even after 96 h of exposure to this compound (data not shown).

Depletion of Pyrimidine Ribonucleotides in CAD Deficient G9c Cells Results in S-Phase Arrest. Finally, to further investigate the requirement for CTP during cell cycle progression, we examined the effects of pyrimidine starvation of a cell line (G9c) lacking CAD, a key enzyme in the de novo pyrimidine synthetic pathway. These cells require supplementation of the media with uridine (30 μM) to allow growth in the absence of a functional de novo pathway (Banerjee and Davidson, 1997). Similar to the LEF results, uridine starvation of G9c cells induced a time-dependent S phase arrest that correlated with the decrease in intracellular pyrimidine ribonucleotide pools (Fig. 7A; data not shown). The percentage of cells in S phase rose from 26.5% in the control
G9c cells to 81.0% after 24 h of starvation, whereas the percentage of cells in G2/M phase decreased rapidly from 9.4% in the control cells to 1.4% followed by starvation of uridine for 24 h. The cells remained in S phase after 24 h starvation of uridine (Fig. 7A) and approximately 40% of these cells underwent apoptosis after 48 h of uridine starvation.

Similar to the results in K562 cells, both the cell cycle arrest and apoptosis induced by uridine starvation were completely prevented by the addition of cytidine (100 μM). The addition of 30 μM uridine and 100 μM cytidine back to the medium for an additional 24 h largely reversed the S phase arrest induced by uridine starvation (Fig. 7B). The fact that cytidine addition alone reversed the effects of uridine starvation supported the LEF results and further demonstrated that CTP plays a critical role determining pyrimidine ribonucleotide-dependent cell cycle arrest. As observed after LEF treatment, the cell cycle arrest induced by uridine starvation was not prevented by the addition of exogenous thymidine (Fig. 7B).

Discussion

The results of the current investigation demonstrate three important findings: one, that LEF is an effective inducer of erythroid cell differentiation; two, that this occurs through the selective depletion of pyrimidines; and three, that the cellular CTP pool is essential in determining cell cycle progression and cell proliferation. Furthermore, these studies suggest that regulation of pyrimidine synthesis (through either the salvage or de novo pathways) may be an important determinant of cell differentiation (see Fig. 8). In support of these statements, we found that the effects of LEF on cell cycle arrest, hemoglobin expression, glycophorin A expression, cell proliferation, and differentiation were completely prevented with coinubcation of cells with either uridine or cytidine, and were independent of effects on total protein tyrosine phosphorylation (data not shown). Whereas these studies do not exclude the possibility that LEF inhibits a specific tyrosine phosphorylation event, the concentrations necessary to induce differentiation were significantly lower than those reported to inhibit tyrosine kinases (Cherwinski et al., 1995; Xu et al., 1996). Moreover, the observation that a similar, cytidine-sensitive S-phase arrest was induced by pyrimidine starvation of cells lacking the de novo synthetic pathway strongly argues for the depletion of pyrimidines as a primary mechanism of LEF action.

The surprising finding was the importance of the CTP pool in determining cell cycle progression. Although LEF treatment resulted in the depletion of both UTP and CTP pools, cytidine restored only the CTP pool, demonstrating that the replenishment of the CTP pool alone was sufficient to re-establish cell proliferation. By contrast, cytidine treatment actually...
lowered the UTP pool, presumably through CTP-dependent feedback inhibition of the de novo synthesis pathway (Jones, 1980). The finding that UTP was not increased after cytidine treatment indicates that these cells express little or no cytidine deaminase, an alternative route to the synthesis of UTP (Perignon et al., 1985). These results suggest that although reduced, the UTP levels are sufficient to meet the cells needs for the synthesis of UDP-sugars and other metabolites derived from UTP (Butler and Elling, 1999). Finally, the finding that neither deoxycytidine nor thymidine incubation affected the inhibitory actions of LEF, argues that the synthesis of the deoxyribonucleotides (dCTP, dTTP) was not rate-limiting under these conditions.

The requirement for CTP in cell cycle progression is not known. Increased concentrations of ribonucleotides have been shown in a variety of malignancies with the largest increase occurring in cytidine ribonucleotides (Jackson et al., 1980; Weber, 1983). CTP is the immediate precursor of the activated, energy-rich phospholipid pathway intermediates CDP-diacylglycerol, CDP-ethanolamine, and CDP-choline, which are essential intermediates in phospholipid synthesis during cell cycle progression (Jackowski et al., 2000). Treatment of promyelocytic HL-60 cells with an inhibitor of CTP synthase (e.g., cyclopentenyl cytosine) (Ford et al., 1991), induced both growth inhibition and differentiation of these cells that was accompanied by a pronounced decline in the level of CTP, but not of UTP, ATP, or GTP (Glazer et al., 1986). In related studies, we have observed that cyclopentenyl cytosine also induces a dose-dependent differentiation of K562 cells (M. Huang, Y. H. Wang, M. Collins, and L. M. Graves, unpublished observations). Taken together, these observations suggest that the immunomodulatory effects of LEF may be shared with other compounds that disrupt CTP synthesis in cells.

The results of our studies demonstrated that LEF induced a pronounced S phase arrest that preceded the differentiation of K562 cells and was recapitulated in CAD-deficient, uridine-starved G9c cells, indicating that the effect of LEF was not simply an artifact of drug treatment. Analysis of additional cell cycle parameters supported our flow cytometry data, demonstrating that LEF and uridine starvation of G9c cells induced an S phase and not a G1 phase arrest. Specifically, the steady increase in both Cdk-2 activity and cyclin A expression was consistent with normal progression of cells through the G1 phase of the cell cycle (den Elzen and Pines, 2001). The observation that the cyclin-dependent kinase inhibitor p21cip1, which inhibits the activity of cyclin A- and E-dependent kinases (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993; Adams et al., 1996), was transiently induced and degraded in response to LEF treatment (K562 cells) or uridine starvation (G9c) further supports the flow cytometry data that these cells were not blocked in the G1 phase of the cell cycle. Analogous to the effects that we observed with LEF, depletion of cellular CTP pools by cyclopentenyl cytosine also resulted in a dose-dependent accumulation of cells in S phase in several human and murine tumor cell lines (Agbaria et al., 1997). Exposure of K562 cells to phosphonate 9-(2-phosphonyl-methoxyethyl) adenine, a DNA synthesis inhibitor, resulted in a similar S phase arrest and erythroid differentiation, indicating that duplication of the cellular genome during this phase of the cell cycle is a critical event during which the cells are highly susceptible to the induction of differentiation (Hatse et al., 1999b).

Interestingly, our results differed from earlier studies showing that inhibition of de novo pyrimidine synthesis with LEF (Ruckemann et al., 1998) or N-(phosphonacetyl)-L-aspartate (PALA) cells (Linke et al., 1996) resulted in a G1 phase arrest of normal human T lymphocytes. The fact that we did not observe a G1 phase arrest might reflect the p53 status of the cells used in this study. The effects of PALA on the cell cycle have been shown to be dependent on p53 expression (Agarwal et al., 1998); mammalian cell lines TR9–7, W138 expressing normal levels of p53, arrested in G1 in response to PALA. In comparison, a p53 mutant human fibroblast cell line MDAH041 as well as cell lines C11 and REF52 that retain a low level of wild-type p53, arrested in S phase in response to PALA (Agarwal et al., 1998). We observed a pyrimidine-dependent S phase arrest in both uridine-starved CAD-deficient G9c cells or LEF-treated K562 cells. The p53 status in both K562 cells and the parental cell line of G9c (Chinese hamster ovary K1) are mutant as a result of a frameshift mutation (Law et al., 1993) or a Thr-to-Lys mutation (Hu et al., 1999), respectively. Thus, these observations are consistent with a p53-independent cell cycle progression.
(S phase) checkpoint that responds to pyrimidine ribonucleotide starvation.

Finally, these studies suggest an important role for pyrimidine synthesis in determining cell proliferation, differentiation, or death. We have recently observed that CAD, the rate-limiting enzyme in the de novo synthesis of pyrimidine, is inactivated and degraded during the differentiation of muscle myoblasts (C2C12) (D. Shea, M. Huang, L. M. Graves, unpublished observations) and apoptosis of 32D cells, events that are paralleled by a specific loss of pyrimidines (Huang et al., 2002). Depletion of pyrimidines with PALA or LEF (as shown in this study) can also induce apoptosis, suggesting that inadequate levels of these ribonucleotides may undermine cell viability. Moreover, the current studies raise cautions regarding pharmacological approaches (e.g., LEF) designed to inhibit the de novo pyrimidine synthesis. Given our

Fig. 7. Effects of pyrimidine starvation on cell cycle progression in CAD-deficient cells. A, CAD-deficient (G9c) cells were cultured in medium without uridine for times indicated. B, CAD-deficient G9c cells were starved with uridine in the absence or presence of cytidine and thymidine, respectively, for times indicated. Alternatively, 30 μM uridine, 100 μM cytidine, or 100 μM thymidine was added for an additional 24 h after deprivation of uridine for 24 h (bottom three on the right). The cells were then stained with propidium iodide and were analyzed for DNA content by flow cytometry. A total of 20,000 cells analyzed from each sample, and the percentage of cells in G1, S, and G2/M phases of cell cycle was determined.
data that the effects of LEF were prevented by either uridine or cytidine addition and that considerable quantities of these nucleosides are continuously present in plasma (Traut, 1994), compensatory synthesis through the salvage pathway must also be considered (Fig. 8).

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