0026-895X/02/6203-463–472$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 62:463–472, 2002

Vol. 62, No. 3
1710/1002908
Printed in U.S.A.

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

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Received March 1, 2002; accepted March 22, 2002

This article is available online at http://molpharm.aspetjournals.org

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 deoxycytidine 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; Pinschewer 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 IC_{50} values for growth inhibition of various mammalian cells and the effects of LEF on the murine leukemia cell line LSTRA (IC_{50} = 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-

This work was supported by National Institutes of Health grants R01-GM59767 (to L.M.G.), R01-CA34085 (to B.S.M.), an AHA EI grant (to L.M.G.), and a Leukemia Research Foundation grant (to M.H.).

ABBREVIATIONS: RA, rheumatoid arthritis; LEF, leflunomide; CAD, carbamoyl phosphate synthetase/aspartate transcarbamoylase/dihydroorotase; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PE, phycoerythrin; PALA, *N*-(phosphonacetyl)-L-aspartate; CDK, cyclin-dependent kinase.

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 when treated with nucleoside analogs that interfere with DNA replication such as 1-β-D-arabinofuranosylcytosine (Bianchi Scarra et al., 1986) and 5-azacytidine (Gambari et al., 1984).

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% CO_2. 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-glycophorin A phycoerythrin (PE) was obtained from BD Biosciences, San Jose, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from PerkinElmer Life Science (Boston, MA).

Cell Cycle Analysis. Cells (5×10^6) were fixed in 5 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 RNAase 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 FACScan flow cytometer (BD Biosciences, San Jose, CA) and percentage of cells within the G_1, S, and G_2/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 H_2O_2 (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 (1.0×10^7) 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 $NH_4H_2PO_4$, pH 3.8, and the elution buffer (buffer B) contained 250 mM $NH_4H_2PO_4$, 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 Glycophorin A Expression. Untreated and drug-exposed K562 cells (1×10^6 cells/sample) were collected and washed twice with PBS, then resuspended in 200 μl of PBS. PE (20 μl)-conjugated antibodies against glycophorin 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 FACScan 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 Na_3VO_4, 0.25 mM phenylmethysulfonyl fluoride, 5 μg/ml leupeptin, and 10 nM 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-polyacylamide gel electrophoresis and transferred to

polyvinylidene difluoride membranes (Immobilon P; Millipore Corporation, Bedford, MA). The membrane was blocked with 3% gelatin in 0.1% Tween 20 in TBS for 1 h and then incubated with primary antibody. Primary antibodies against p21, Cdk2, cyclin A and cyclin E, were used at a dilution of 1:1,000 in blocking solution and the membrane was then washed three times with Tween 20 in TBS and incubated in appropriate secondary antibody (1:5,000 dilution of horseradish peroxidase-linked anti-rabbit or anti-mouse immunoglobulin (Santa Cruz Biochemicals, Santa Cruz, CA). Detections were performed using the ECL chemiluminescence system (Amersham Biosciences).

Immunoprecipitation and Cdk2 Kinase Assay. Cells were washed twice with ice-cold PBS, and then lysed in a lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol containing 2 mM EDTA, 150 μM Na_3VO_4, 0.25 mM phenylmethysulfonyl fluoride, 5 μg/ml leupeptin, and 10 nM microcystin LR). After a brief sonication on ice, lysates were cleared by centrifugation, and 300 μg of proteins of the supernatant was mixed with lysis buffer to increase the volume to 300 μl. α-Cdk2 (M2) rabbit polyclonal IgG Ab (5 μl; sc-163; Santa Cruz Biotechnology, Santa Cruz, CA) was added and incubated for 2 h at 4°C on a rotator. Protein A-agarose bead (25 μl; 50%, v/v) was added to the mixture and incubated for 1h at 4°C on a rotator. Immune complexes were collected by centrifugation at 12,000 rpm for 5 min at 4°C, and the beads were washed three times with lysis buffer, and once with kinase buffer (50 mM HEPES, pH 7.3, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM EGTA, 20 μM ATP, 10 mM β-glycerophosphate, 1 mM NaF). The immune complexes were then incubated with 25 μl of kinase reaction mixture containing 50 mM HEPES pH 7.3, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM EGTA, 20 μM ATP, 10 mM β-glycerophosphate, 1 mM NaF, 1.5 μg of histone H1, and 5 μCi of [γ-^{32}P]ATP for 30 min at 37°C. After centrifugation at 12,000 rpm for 1 min, 20 μl of supernatants were spotted on P-81 paper (Whatman), washed five times in 150 mM phosphoric acid, and counted for radioactivity in a liquid scintillation counter.

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 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).

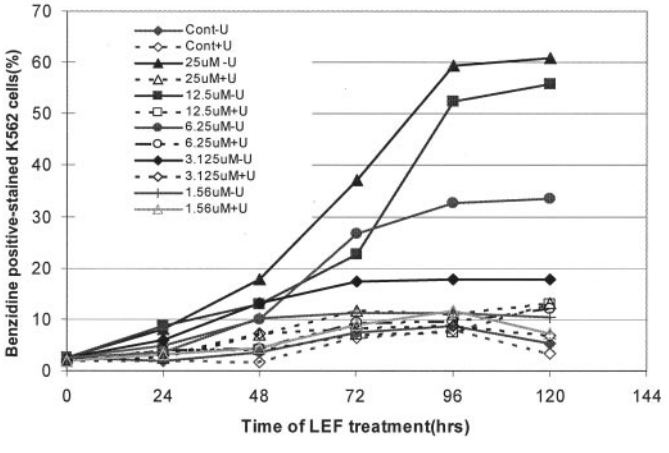

Fig. 1. Effect of LEF on differentiation of K562 erythroleukemic cells. K562 cells were exposed to LEF at concentrations and times indicated. The percentage of hemoglobin containing cells that stained positive for benzidine were obtained by counting at least 1000 cells per sample under microscopy using 100× magnification (data represent one of the three representative experiments). Shown is a plot representative of three experiments.

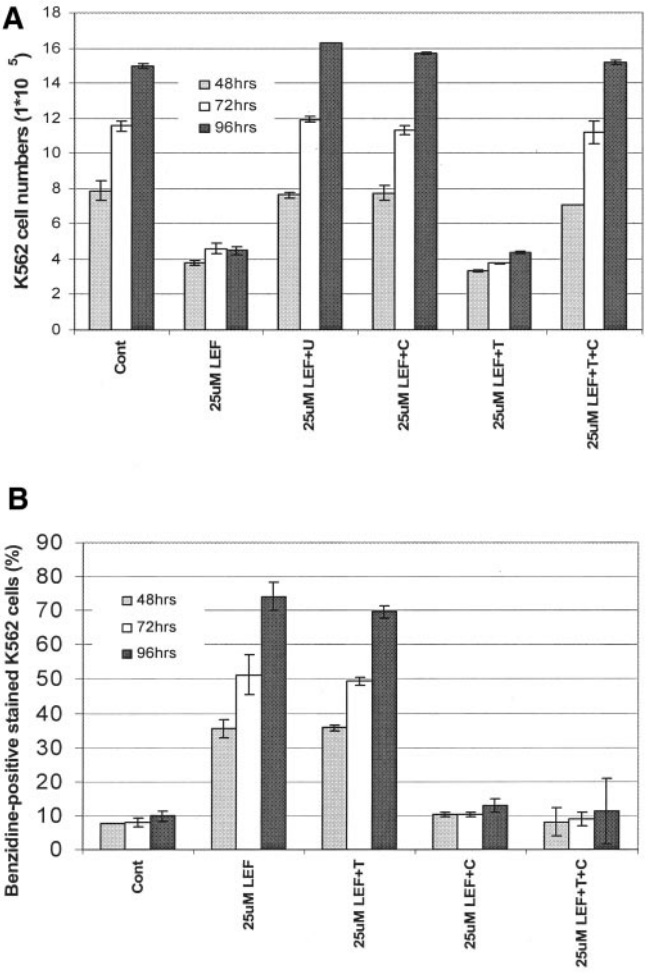

Fig. 2. Effects of uridine, cytidine, or thymidine on LEF-induced growth inhibition and differentiation of K562 cells. Cells were cultured in the absence (control) or presence of 25 μM LEF or with 25 μM LEF plus 30 μM uridine, 100 μM cytidine, or 100 μM thymidine, respectively, at times indicated. A, 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.

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 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

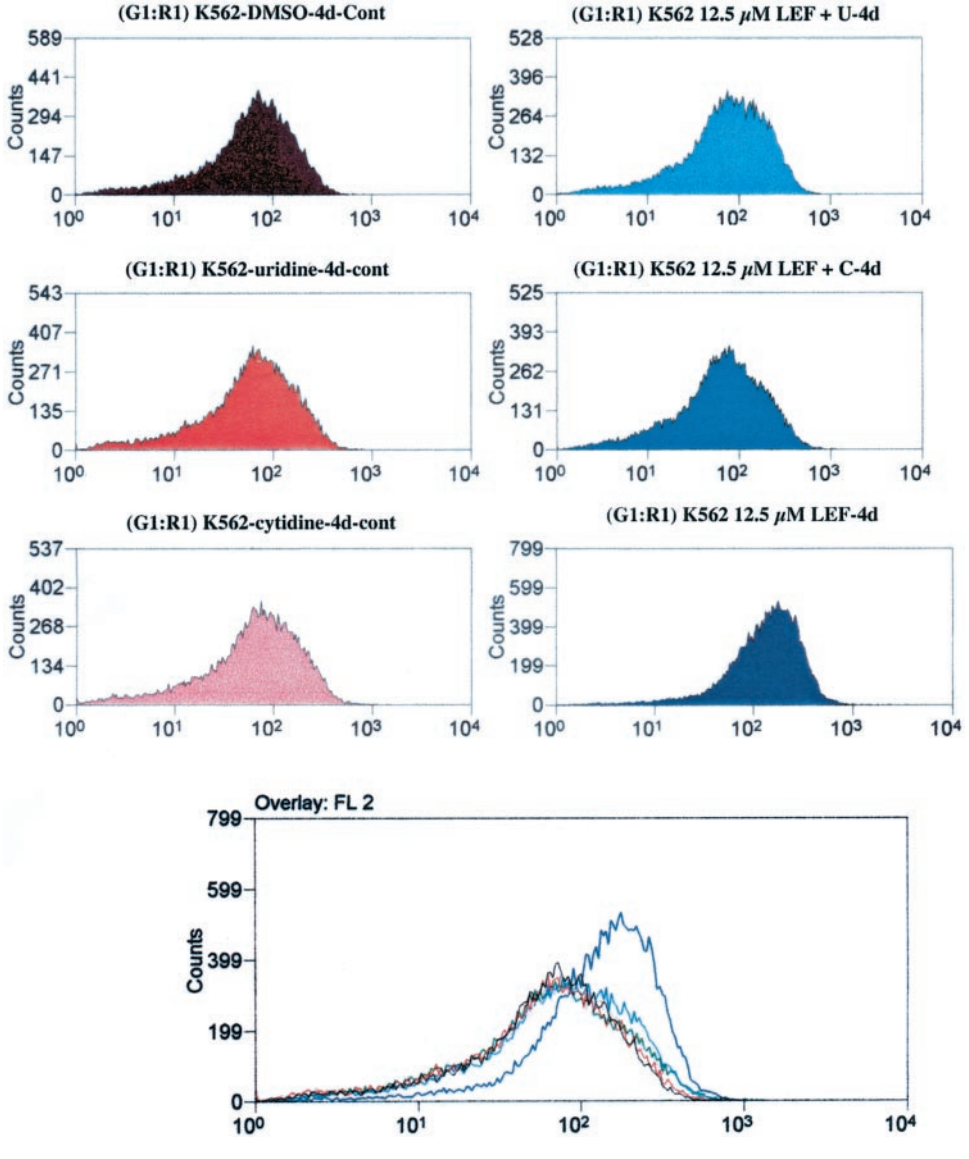

Fig. 3. Effect of uridine and cytidine on glycophorin A expression in K562 cells. After a 4-day exposure to LEF (12.5 μM), in the presence or absence of 30 μM uridine or 100 μM cytidine, K562 cells were stained with PE-conjugated monoclonal antibody against glycophorin A. The fluorescence of the stained cells was measured by flow cytometry as described under *Materials and Methods*.

17%, respectively, of the corresponding K562 control cells (Fig. 4B). Addition of uridine to these cells (24h) resulted in a marked increase in both the UTP and CTP pools (136 and 195% of control, respectively) but had little or no effect on the cellular purine pools (ATP and GTP). By comparison, cytidine addition increased only CTP (600% of control) and caused a slight decrease of cellular UTP and purine pools in untreated K562 cells. Coincubation of LEF-treated cells with uridine increased UTP and CTP pools (236 and 187% of control), indicating that the pyrimidine salvage pathway successfully restored the cellular pyrimidine pools after LEF exposure. Interestingly, cytidine effectively restored the CTP pools but failed to restore UTP pools after LEF-treatment (Fig. 4B), suggesting that the effects of cytidine addition were specifically mediated through changes in CTP synthesis.

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, G_1-, S-, and G_2-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 G_2-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).

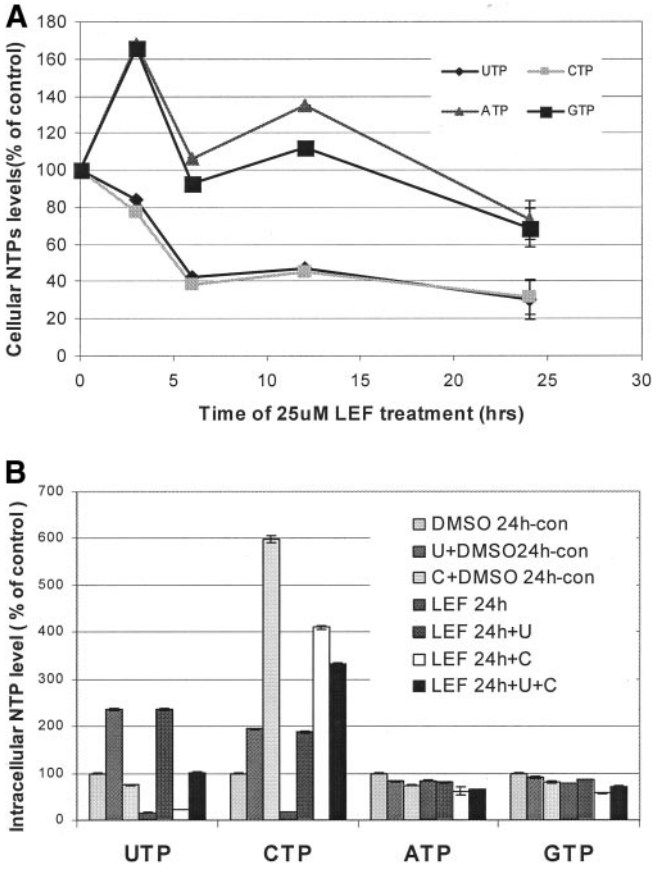

Fig. 4. Depletion of cellular pyrimidine pools by LEF exposure and effects of exogenous uridine and cytidine on cellular nucleotide levels. A, cells were exposed to 25 μM LEF at times indicated. B, cells were cultured in the absence (vehicle control) or presence of 25 μM LEF or with 25 μM LEF plus 30 μM uridine or 100 μM cytidine at times indicated. A and B, intracellular UTP, CTP, GTP, and ATP were extracted and measured as described under *Materials and Methods* and shown as the percentage of nucleotide remaining after exposure to LEF compared with the vehicle control. Samples were corrected for cell numbers and the values represent the mean of duplicate samples.

To eliminate the possibility that these results were mediated through the depletion of pyrimidine deoxynucleotide pools, the effects of exogenous deoxycytidine and thymidine on LEF induced cell cycle arrest of K562 cells was also investigated. As shown in Fig. 5, addition of 100 μM thymidine failed to reverse the cell cycle arrest induced by LEF exposure at 24h, but showed a slight effect on cell cycle procession after 48h. Similarly, the addition of deoxycytidine did not prevent the LEF-induced cell cycle arrest or differentiation of K562 cells (data not shown), demonstrating that the effects of LEF were not mediated through changes in the pyrimidine deoxynucleotide pools.

Effect of LEF on the Expression of Cell Cycle-Regulatory Proteins in K562 Cells. Several key regulators of S phase initiation, procession, and termination were examined after LEF treatment. The expression of Cdk-2, cyclin A, cyclin E, and p21 were examined by immunoblotting lysates 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 compared 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 (Banerjei 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 G_2/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 coincubation 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 repletion of the CTP pool alone was sufficient to re-establish cell proliferation. By contrast, cytidine treatment actually

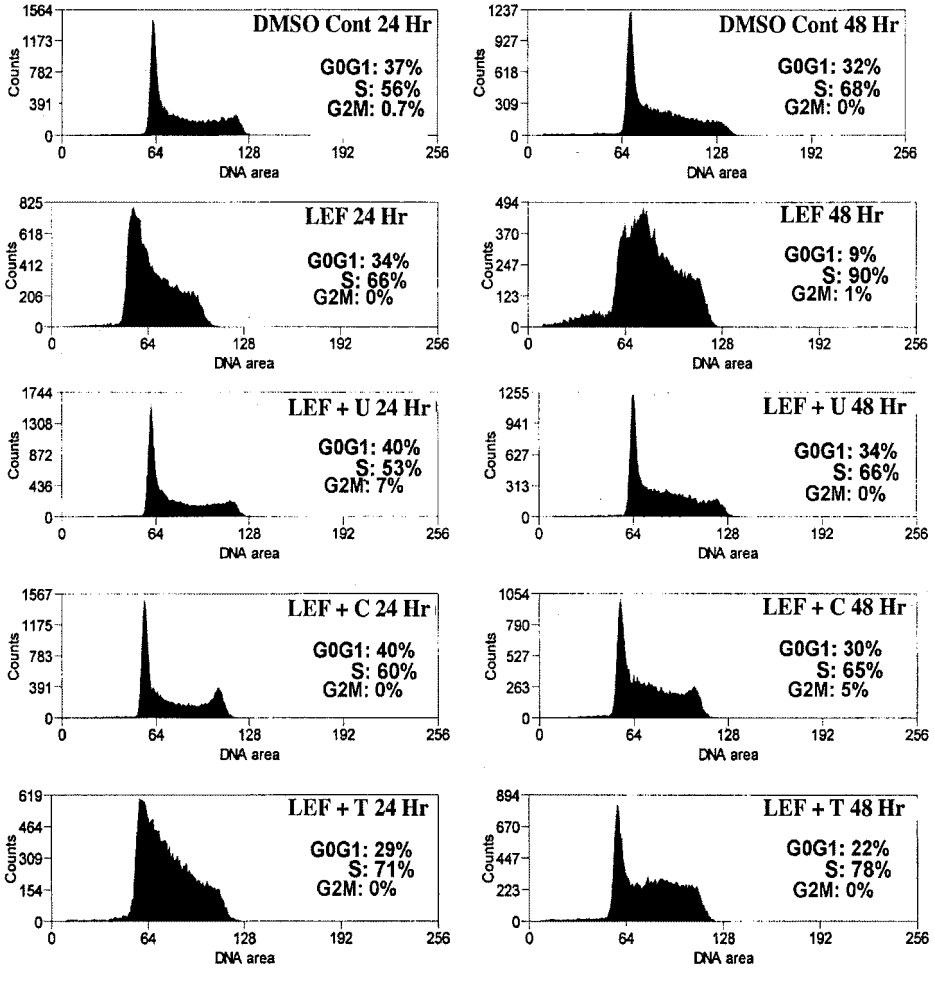

Fig. 5. Effect of exogenous uridine, cytidine, or thymidine on LEF-induced cell cycle arrest of K562 cells. K562 cells were treated in the absence (control) or presence of 25 μM LEF or with 25 μM LEF plus 30 μM uridine, 100 μM cytidine, or 100 μM thymidine, respectively, for times indicated. Cells were then stained with propidium iodide and were analyzed for DNA content by flow cytometry. A total of 20,000 cells was analyzed from each sample, and the percentage of cells in G_1, S, and G_2/M phases of cell cycle was determined.

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.

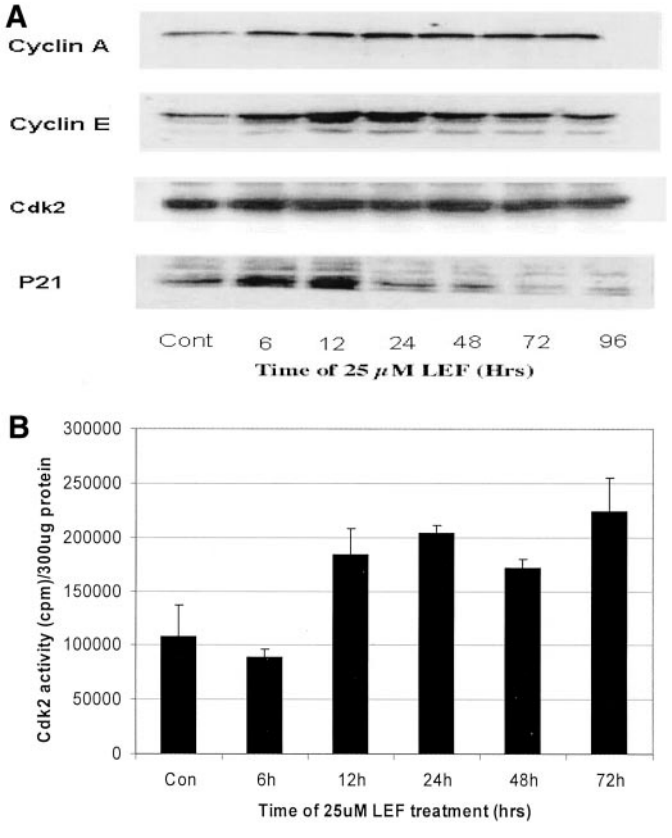

Fig. 6. Effects of LEF on cell cycle regulating proteins in K562 cells. A, K562 cells were treated with 25 μM LEF or with vehicle for indicated times, and expressions of cell cycle proteins were assessed by immunological staining and ECL detection. P21, Cdk2, cyclin E, and cyclin A migrated at molecular masses of 21,000, 33,000, 50,000/52,000, and 60,000, respectively. Lane 1, untreated control; lanes 2 to 7, 25 μM LEF at 6, 12, 24, 48, 72, and 96 h, respectively. K562 cells were treated with 25 μM LEF or with vehicle for indicated times. B, cell lysates (300 μg) were immunoprecipitated with antibodies to Cdk2 (M2) and the immunoprecipitated proteins were incubated with Cdk2 reaction buffer containing substrate histone H1, and the Cdk2 activity was measured as described under *Materials and Methods*. Results are presented as the mean ± S.D. from two experiments conducted in duplicate.

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 G_1 phase arrest. Specifically, the steady increase in both Cdk-2 activity and cyclin A expression was consistent with normal progression of cells through the G_1 phase of the cell cycle (den Elzen and Pines, 2001). The observation that the cyclin-dependent kinase inhibitor $p21^{cip1,waf-1}$, 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 G_1 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 G_1 phase arrest of normal human T lymphocytes. The fact that we did not observe a G_1 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 G_1 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

(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

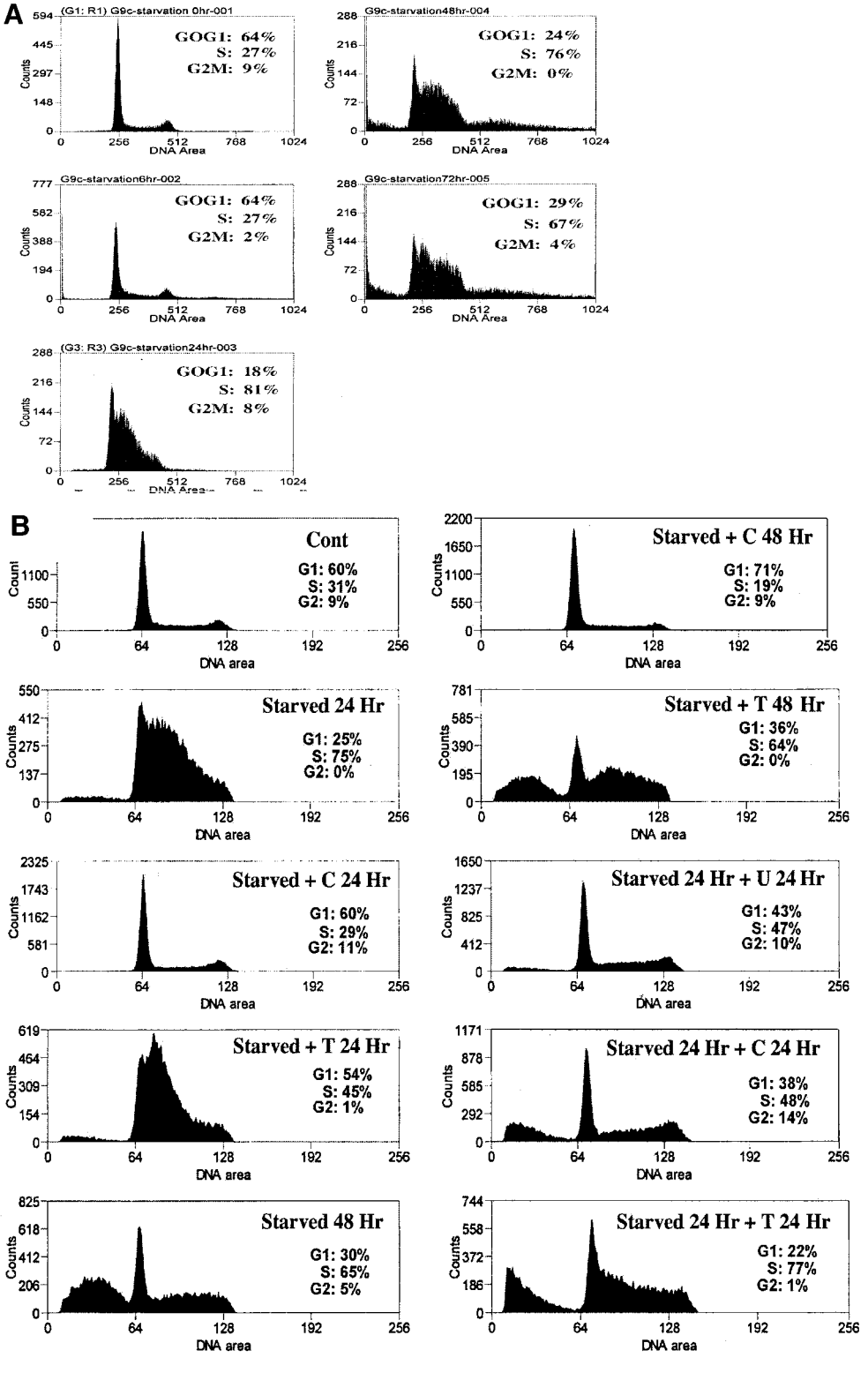

Fig. 7. Effects of pyrimidine starvation on cell cycle progression in CAD-deficient cells. A, CAD-deficient (G9c) cells were cultured in medium without uridine for times indicated. B, CAD-deficient G9c cells were starved with uridine in the absence or presence of cytidine and thymidine, respectively, 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 G_1, S, and G_2/M phases of cell cycle was determined.

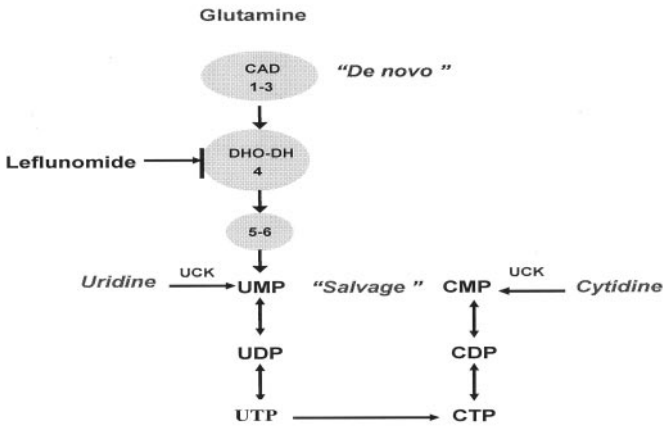

Fig. 8. Model of pyrimidine synthesis by de novo and salvage pathways. A model is depicted showing the de novo and salvage pyrimidine nucleotides synthesis in mammalian cells (Jones, 1980; Perignon et al., 1985; Traut, 1994). The de novo synthesis of pyrimidine nucleotides requires 6 enzymes. The first three (1–3) are catalyzed by a trifunctional, cytoplasmic enzyme known as CAD, an acronym derived from the names of the three activities in this protein, Carbamoyl phosphate synthetase, Aspartate transcarbamylase, and Dihydroorotase. The fourth enzyme (4) is a mitochondrial enzyme, dihydroorotate dehydrogenase. The last two steps (5–6) are cytoplasmic and are catalyzed by the bifunctional enzyme orotate phosphoribosyltransferase/orotidine-5′-monophosphate decarboxylase. The proposed site of LEF inhibition in the de novo biosynthetic pathway is illustrated and the salvage pathway synthesis from uridine and cytidine is catalyzed by uridine/cytidine kinase (UCK).

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).

Acknowledgments

Dr. Thomas Traut is acknowledged for critical evaluation of the manuscript. We thank Dr. David Evans for the gift of the G9c cells.

References

Adams PD, Sellers WR, Sharma SK, Wu AD, Nalin CM, and Kaelin WG Jr (1996) Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. *Mol Cell Biol* **16:**6623–6633.

Agarwal ML, Agarwal A, Taylor WR, Chernova O, Sharma Y, and Stark GR (1998) A p53-dependent S-phase checkpoint helps to protect cells from DNA damage in response to starvation for pyrimidine nucleotides. *Proc Natl Acad Sci USA* **95:** 14775–14780.

Agbaria R, Kelley JA, Jackman J, Viola JJ, Ram Z, Oldfield E, and Johns DG (1997) Antiproliferative effects of cyclopentenyl cytosine (NSC 375575) in human glioblastoma cells. *Oncol Res* **9:**111–118.

Banerjei LC and Davidson JN (1997) Site-directed substitution of Ser1406 of hamster CAD with glutamic acid alters allosteric regulation of carbamyl phosphate synthetase II. *Somat Cell Mol Genet* **23:**37–49.

Bianchi Scarra GL, Romani M, Coviello DA, Garre C, Ravazzolo R, Vidali G, and Ajmar F (1986) Terminal erythroid differentiation in the K-562 cell line by 1-beta-D-arabinofuranosylcytosine: accompaniment by c-myc messenger RNA decrease. *Cancer Res* **46:**6327–6332.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Butler T and Elling L (1999) Enzymatic synthesis of nucleotide sugars. *Glycoconj J* **16:**147–159.

Cherwinski HM, Cohn RG, Cheung P, Webster DJ, Xu YZ, Caulfield JP, Young JM, Nakano G, and Ransom JT (1995) The immunosuppressant leflunomide inhibits lymphocyte proliferation by inhibiting pyrimidine biosynthesis. *J Pharmacol Exp Ther* **275:**1043–1049.

den Elzen N and Pines J (2001) Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. *J Cell Biol* **153:**121–136.

Elder RT, Xu X, Williams JW, Gong H, Finnegan A, and Chong AS (1997) The immunosuppressive metabolite of leflunomide, A77 1726, affects murine T cells through two biochemical mechanisms. *J Immunol* **159:**22–27.

Fairbanks LD, Bofill M, Ruckemann K, and Simmonds HA (1995) Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. Disproportionate expansion of pyrimidine pools and contrasting effects of de novo synthesis inhibitors. *J Biol Chem* **270:**29682–9.

Ford H Jr, Cooney DA, Ahluwalia GS, Hao Z, Rommel ME, Hicks L, Dobyns KA, Tomaszewski JE, and Johns DG (1991) Cellular pharmacology of cyclopentenyl cytosine in Molt-4 lymphoblasts. *Cancer Res* **51:**3733–3740.

Fox RI, Herrmann ML, Frangou CG, Wahl GM, Morris RE, Strand V, and Kirschbaum BJ (1999) Mechanism of action for leflunomide in rheumatoid arthritis. *Clin Immunol* **93:**198–208.

Gambari R, del Senno L, Barbieri R, Viola L, Tripodi M, Raschella G, and Fantoni A (1984) Human leukemia K-562 cells: induction of erythroid differentiation by 5-azacytidine. *Cell Differ* **14:**87–97.

Ghosh S, Zheng Y, Jun X, Narla RK, Mahajan S, Navara C, Mao C, Sudbeck EA, and Uckun FM (1998) Alpha-cyano-beta-hydroxy-beta-methyl-*N*-[4-(trifluoromethoxy-)phenyl] propenamide: an inhibitor of the epidermal growth factor receptor tyrosine kinase with potent cytotoxic activity against breast cancer cells. *Clin Cancer Res* **4:**2657–2668.

Glazer RI, Cohen MB, Hartman KD, Knode MC, Lim MI, and Marquez VE (1986) Induction of differentiation in the human promyelocytic leukemia cell line HL-60 by the cyclopentenyl analogue of cytidine. *Biochem Pharmacol* **35:**1841–1848.

Gu Y, Turck CW, and Morgan DO (1993) Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature (Lond)* **366:**707–710.

Harper JW, Adami GR, Wei N, Keyomarsi K, and Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75:**805–816.

Hatse S, De Clercq E, and Balzarini J (1999a) Role of antimetabolites of purine and pyrimidine nucleotide metabolism in tumor cell differentiation. *Biochem Pharmacol* **58:**539–555.

Hatse S, Schols D, De Clercq E, and Balzarini J (1999b): 9-(2-Phosphonylmethoxyethyl)adenine induces tumor cell differentiation or cell death by blocking cell cycle progression through the S phase. *Cell Growth Differ* **10:**435–446.

Herrmann ML, Schleyerbach R, and Kirschbaum BJ (2000) Leflunomide: an immunomodulatory drug for the treatment of rheumatoid arthritis and other autoimmune diseases. *Immunopharmacology* **47:**273–289.

Hu T, Miller CM, Ridder GM, and Aardema MJ (1999) Characterization of p53 in Chinese hamster cell lines CHO-K1, CHO-WBL and CHL: implications for genotoxicity testing. *Mutat Res* **426:**51–62.

Huang M, Kozlowski P, Collins M, Wang Y, Haystead TA, and Graves LM (2002) Caspase-dependent cleavage of carbamoyl phosphate synthetase II during apoptosis. *Mol Pharmacol* **61:**569–577.

Jackowski S, Wang J, and Baburina I (2000) Activity of the phosphatidylcholine biosynthetic pathway modulates the distribution of fatty acids into glycerolipids in proliferating cells. *Biochim Biophys Acta* **1483:**301–315.

Jackson RC, Lui MS, Boritzki TJ, Morris HP, and Weber G (1980) Purine and pyrimidine nucleotide patterns of normal, differentiating and regenerating liver and of hepatomas in rats. *Cancer Res* **40:**1286–1291.

James SJ, Miller BJ, Basnakian AG, Pogribny IP, Pogribna M, and Muskhelishvili L (1997) Apoptosis and proliferation under conditions of deoxynucleotide pool imbalance in liver of folate/methyl deficient rats. *Carcinogenesis* **18:**287–293.

Jones ME (1980) Pyrimidine nucleotide biosynthesis in animals: genes, enzymes and regulation of UMP biosynthesis. *Annu Rev Biochem* **49:**253–279.

Law JC, Ritke MK, Yalowich JC, Leder GH, and Ferrell RE (1993) Mutational inactivation of the p53 gene in the human erythroid leukemic K562 cell line. *Leuk Res* **17:**1045–1050.

Linke SP, Clarkin KC, Di Leonardo A, Tsou A, and Wahl GM (1996) A reversible, p53-dependent G_0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Genes Dev* **10:**934–947.

Lozzio CB and Lozzio BB (1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* **45:**321–334.

Mahajan S, Ghosh S, Sudbeck EA, Zheng Y, Downs S, Hupke M, and Uckun FM (1999) Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-*N*-(2, 5-dibromophenyl)propenamide]. *J Biol Chem* **274:**9587–9599.

Nagy K, Pasti G, Bene L, and Nagy I (1995) Involvement of Fenton reaction products in differentiation induction of K562 human leukemia cells. *Leuk Res* **19:**203–212.

Nair RV, Cao W, and Morris RE (1995) Inhibition of smooth muscle cell proliferation in vitro by leflunomide, a new immunosuppressant, is antagonized by uridine. *Immunol Lett* **48:**77–80.

Perignon JL, Chaleon J, Leverger G, Houllier AM, Thuillier L, and Cartier PH (1985) Cytidine deaminase activity of human normal and malignant lymphoid cells. *Clin Chim Acta* **147:**67–74.

Pinschewer DD, Ochsenbein AF, Fehr T, and Zinkernagel RM (2001) Leflunomide-mediated suppression of antiviral antibody and T cell responses: differential restoration by uridine. *Transplantation* **72:**712–719.

Pogolotti AL Jr and Santi DV (1982) High-pressure liquid chromatography—ultraviolet analysis of intracellular nucleotides. *Anal Biochem* **126:**335–345.

Ruckemann K, Fairbanks LD, Carrey EA, Hawrylowicz CM, Richards DF, Kirschbaum B, and Simmonds HA (1998) Leflunomide inhibits pyrimidine de novo synthesis in mitogen-stimulated T-lymphocytes from healthy humans. *J Biol Chem* **273:**21682–21691.

Strawn LM, Kabbinavar F, Schwartz DP, Mann E, Shawver LK, Slamon DJ, and Cherrington JM (2000) Effects of SU101 in combination with cytotoxic agents on the growth of subcutaneous tumor xenografts. *Clin Cancer Res* **6:**2931–2940.

Traut TW (1994) Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem* **140:**1–22.

Weber G (1983) Biochemical strategy of cancer cells and the design of chemotherapy: G. H. A. Clowes Memorial Lecture. *Cancer Res* **43:**3466–3492.

Williamson RA, Yea CM, Robson PA, Curnock AP, Gadher S, Hambleton AB, Woodward K, Bruneau JM, Hambleton P, Moss D, et al. (1995) Dihydroorotate dehydrogenase is a high affinity binding protein for A77 1726 and mediator of a range

of biological effects of the immunomodulatory compound. *J Biol Chem* **270:**22467–72.
Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, and Beach D (1993) p21 is a universal inhibitor of cyclin kinases. *Nature (Lond)* **366:**701–704.
Xu X, Shen J, Mall JW, Myers JA, Huang W, Blinder L, Saclarides TJ, Williams JW, and Chong AS (1999) In vitro and in vivo antitumor activity of a novel immunomodulatory drug, leflunomide: mechanisms of action. *Biochem Pharmacol* **58:** 1405–1413.
Xu X, Williams JW, Gong H, Finnegan A, and Chong AS (1996) Two activities of the immunosuppressive metabolite of leflunomide, A77 1726. Inhibition of pyrimidine nucleotide synthesis and protein tyrosine phosphorylation. *Biochem Pharmacol* **52:**527–534.
Zielinski T, Zeitter D, Muller S, and Bartlett RR (1995) Leflunomide, a reversible inhibitor of pyrimidine biosynthesis? *Inflamm Res* **44(Suppl 2):**S207–S208.

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