Regulation of the salvage pathway of deoxynucleotides synthesis in apoptosis induced by growth factor deprivation

F. Javier OLIVER*, Mary K. L. COLLINS† and Abelardo LÓPEZ-RIVAS*‡

*Instituto de Parasitología y Biomedicina, C. S. I. C., c/Ventanilla 11, 18001 Granada, Spain, and †Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, U.K.

Here we describe changes in dNTP metabolism that precede DNA fragmentation in a model of apoptosis driven by deprivation of the cytokine interleukin 3 (IL-3). In haemopoietic BAF3 cells, IL-3 withdrawal leads to a rapid decrease in the size of dATP, dTTP and dGTP pools without affecting dCTP levels. This imbalance in dNTP pools precedes DNA fragmentation and is accompanied by down-regulation of enzymes controlling the *de novo* and salvage pathways of dNTP synthesis, ribonucleotide reductase and thymidine kinase (TK) respectively. Readdition of IL-3 results in a rapid, protein synthesis-independent restoration of normal dNTP pools, enhanced TK activity and increased precursor incorporation through the

salvage pathway. Up-regulation of TK activity after IL-3 readdition is prevented by the protein kinase C (PKC) inhibitor staurosporin, but not by tyrosine kinase inhibitors. Furthermore activation of PKC by phorbol esters mimics the stimulatory effect of IL-3 on TK activity, suggesting that PKC might be involved in regulating this effect. These results indicate that regulation by IL-3 of the salvage pathway of dNTP synthesis plays a role in the maintenance of cellular dNTP pool balance and suggests that alterations in dNTP metabolism after IL-3 deprivation could be a relevant event in the commitment of haemopoietic cells to apoptosis.

INTRODUCTION

Apoptosis or programmed cell death is a physiological process of cell homeostasis by which unwanted cells are triggered to die [1]. This homeostatic function regulates tissue dynamics, especially in continuously renewing tissues such as the haemopoietic system, where the steady state is achieved by a balance between cell death and cell division [2]. Regenerative haemopoiesis is stimulated by cytokines, including interleukin 3 (IL-3) and granulocyte/macrophage colony-stimulating factor, which act on the earliest precursor cells [3]. Withdrawal of these cytokines from primary cells or cell lines derived from bone marrow leads to a programme of events typical of apoptosis, including chromatin digestion into oligonucleosome-size fragments [4,5]. Although apoptosis has been observed in enucleated cells, and DNA fragmentation is not a prerequisite for other cellular events during apoptosis [6], in haemopoietic BAF3 cells the apoptotic process becomes irreversible at a time when DNA fragmentation is first observed [7]. Little is known of the mechanism that leads to commitment of cells to apoptosis induced by growth factor deprivation. Because protein synthesis is not always required for apoptosis [4], control of this cell death process must be at the level of post-translational regulation. IL-3 promotes cell survival, proliferation and differentiation of haemopoietic cells through several receptor-linked signalling pathways. These include activation of protein kinase C (PKC) [8], tyrosine and serine phosphorylation of several substrates [9] through the ras/raf/mitogen-activated protein kinase signalling pathway [10] or in association with Jak II kinase [11]. However, a mechanism linking the loss of any of these signals with the initiation of apoptosis after cytokine deprivation has not been clearly demonstrated. Different antineoplastic agents, which inhibit DNA precursor synthesis, have been reported to kill cells by a mechanism involving programmed cell death [12–14], and alterations in dNTP pool balance have been suggested as one of

the events leading to cell death [15]. Previous results from our laboratory have shown that inhibition of the enzyme thymidylate synthase with 5-fluoro-2'-deoxyuridine, to perturb deoxyribonucleotide metabolism, drives the haemopoietic cell line BAF3 to an apoptotic programme, even in the presence of IL-3 [14]. In the present study we show that in haemopoietic cells IL-3 withdrawal leads to a rapid imbalance in dNTP pools that precedes DNA fragmentation, similar to that observed when apoptosis is triggered by treating the cells with inhibitors of dNTP *de novo* synthesis. We also demonstrate that the salvage pathway of dNTP synthesis, particularly thymidine kinase (TK) activity, is regulated by IL-3 and is important in the control of cellular dNTP pools in haemopoietic cells.

MATERIALS AND METHODS

Materials

RPMI 1640 medium and fetal bovine serum were obtained from Gibco. Deoxy[8-³H]adenosine 5'-triphosphate (24 Ci/mmol), [¹⁴C]adenosine 5'-diphosphate, deoxy[5-³H]guanosine 5'-triphosphate, deoxy[5-³H]cytidine 5'-triphosphate and [methyl-³H]thymidine (49 Ci/mmol) were from Amersham; [methyl-³H]thymidine 5'-triphosphate (53 Ci/mmol) and [5,6-³H]uridine were obtained from ICN Biomedicals. Other reagents of the purest grade available were obtained from Sigma and Boehringer-Mannheim. Murine IL-3 was from Peprotech.

Cell cultures

Murine IL-3-dependent BAF3 cells [16] were maintained in culture in RPMI medium containing 10% (w/v) fetal bovine serum, 1 mM glutamine and 10% conditioned medium from the IL-3-producing cell line Wehi-3B (Wehi-3B-CM). Cell viability was determined by Trypan Blue dye exclusion.

Abbreviations used: IL-3, interleukin 3; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; RNR, ribonucleotide reductase; TK, thymidine kinase; Wehi-3B-CM, conditioned media from Wehi-3B cells.

‡ To whom correspondence should be addressed.

Analysis of DNA fragmentation

Fragmented DNA was obtained and analysed as described in [17].

Deoxyribonucleoside triphosphate pool assay

Preparation of cell extract was essentially as described previously [18] with slight modifications. Briefly, $(2-5) \times 10^6$ BAF3 cells were washed with ice-cold PBS. The pellet was resuspended in 50 μ l of PBS and cells were lysed with 0.5 M HClO₄ for 30 min on ice. Precipitated material was discarded after centrifugation and the supernatant was neutralized with 4 M KOH/0.4 M KH₂PO₄ and kept at -20°C until use after discarding the undissolved salt. Deoxyribonucleotides were determined by a modification of the DNA polymerase assay [19]. The concentrations of dNTP were estimated from calibration curves of known picomole amounts of pure standards.

Ribonucleotide reductase (RNR) activity

Enzyme preparations containing a minimum of 2 mg/ml protein were used to assay RNR activity. BAF3 cells were cultured in the presence or the absence of IL-3 (Wehi-3B-CM), as indicated. Cells were pelleted and washed twice with ice-cold PBS. The pellet was then resuspended in 20 mM Tris/HCl, pH 7.0, 2 mM MgCl₂, 20 mM dithiothreitol, 1 mM PMSF, subjected to Dounce homogenization (50 strokes), and sonicated to disrupt the cells completely. The extract was cleared of cellular debris by centrifugation (15000 *g*, 15 min at 4°C) and was assayed for RNR activity essentially as described [20]. Briefly, the standard reductase assay mixture contained as final concentrations: 7.7 mM potassium phosphate buffer, pH 7.0, 19 μ M [¹⁴C]ADP (17.4 mCi/mmol), 5.7 mM dithiothreitol, 10 mM dGTP, 3.9 mM magnesium acetate and 100 μ l of protein extract (0.2–0.5 mg of protein). Enzyme reactions were initiated by addition of extract, carried out for 1 h and terminated by boiling for 5 min. Carrier dAMP (0.3 μ mol) and fresh snake venom suspension (1 mg of *Crotalus adamanteus* venom) was then added, incubated for 3 h at 37°C then boiled for 5 min. Under these conditions reaction mixtures containing [¹⁴C]ADP or [¹⁴C]dATP were quantitatively hydrolysed to their respective nucleosides. Deoxyadenosine was separated from adenosine by using a Dowex-1-borate column as described [20].

TK activity

For cell extract preparation, cells were pelleted and washed twice with ice-cold PBS. The pellet was then resuspended in 20 mM Tris/HCl, pH 7.0, 2 mM MgCl₂, 20 mM dithiothreitol, 1 mM PMSF, subjected to Dounce homogenization and sonicated to disrupt the cells completely. The extract was cleared of cellular debris by centrifugation as before and the remaining supernatant containing a minimum of 2 mg/ml protein was assayed for TK activity essentially as described [21].

Incorporation of uridine

Cells were incubated with [5,6-³H]uridine as precursor (0.3 μ M; 8.3 μ Ci/ μ mol) and samples of 0.2 to 0.4×10^6 cells were taken at different times, washed with ice-cold PBS, resuspended in 10% (w/v) trichloroacetic acid and the radioactivity incorporated into the acid-soluble pool was determined.

Statistical analysis

The statistical significance of the data was determined after applying Student's *t*-test.

RESULTS

dNTP pools imbalance after IL-3 withdrawal

Because we had previous evidence that an imbalance in the dNTP pools caused by inhibitors of dihydrofolate reductase and thymidylate synthetase [14] and RNR activities (results not shown) was sufficient to induce apoptosis in BAF3 cells, our aim was to analyse dNTP metabolism under a more physiological situation known to induce programmed cell death, such as cytokine withdrawal. BAF3 cells were either deprived of IL-3 or maintained in culture in the presence of the cytokine (Wehi-3B-CM). IL-3 removal from BAF3 cells induced DNA fragmentation into oligonucleosome-size fragments representative of apoptosis [4,7] and this endonuclease activation was detected as early as 8 h after cytokine withdrawal (Figure 1A). Analysis of dNTP pools revealed that in IL-3-deprived cells, dTTP pool size was reduced to 50% of the initial level only 3 h after growth factor removal; after 24 h, dTTP was almost completely lost (Figure 1B). The kinetics of dGTP loss was similar to that of dTTP, decreasing to

Figure 1 Effect of IL-3 withdrawal on DNA fragmentation and the size of dNTP pools

(A) DNA was extracted and subjected to agarose gel electrophoresis. Lanes 1–6 correspond to 0, 3, 5, 8, 15 and 24 h after IL-3 deprivation respectively. (B) At the times stated in (A), the levels of the different dNTPs were determined in parallel cultures, as described in the Materials and methods section. Results are the percentages of the initial levels of the different dNTPs and represent the means $\pm$ S.E.M. for two separate experiments in duplicate for dCTP (51.0 ± 4.8 pmol per 10^6 cells) (■) and dGTP (4.8 ± 0.5 pmol per 10^6 cells) (○) and four separate experiments for dATP (6.2 ± 0.6 pmol per 10^6 cells) (●) and dTTP (22.2 ± 1.7 pmol per 10^6 cells) (□).

Table 1 Effect of IL-3 on the recovery of dTTP, dATP and dGTP levels

BAF3 cells were deprived of IL-3 for 6 h and restimulated with the cytokine for 1 h in the presence or the absence of 10 μ g/ml cycloheximide. Perchloric extracts were prepared from the different groups of cells and dNTP was measured. Abbreviations used: IL-3, control cells cultured with IL-3 (Wehi-3B-CM); no IL-3, deprived of IL-3 for 6 h; IL-3 readd., IL-3 (Wehi-3B-CM) readdition for 1 h after 6 h of cytokine deprivation; IL-3 readd. + CHX, as the previous condition but incubated in the presence of 10 μ g/ml cycloheximide during cytokine readdition. dTTP, dATP and dGTP levels were determined as described in the caption to Figure 1. Results are in pmol per 10^6 cells, and are means $\pm$ S.E.M. for three separate experiments (for dTTP and dATP) or two separate experiments (for dGTP), performed in duplicate.

Condition	dTTP	dATP	dGTP
IL-3	32 $\pm$ 2.7	6.6 $\pm$ 0.5	3.4 $\pm$ 0.7
No IL-3	14 $\pm$ 1.0	1.7 $\pm$ 0.3	0.9 $\pm$ 0.3
IL-3 readd.	35 $\pm$ 2.7	4.0 $\pm$ 0.7	3.9 $\pm$ 0.9
IL-3 readd. + CHX	33 $\pm$ 2.7	4.6 $\pm$ 0.7	4.5 $\pm$ 0.1

Table 2 Effect of IL-3 on RNR activity

BAF3 cells were deprived of IL-3 (Wehi-3B-CM) for 6 h and then restimulated with IL-3 for 1 h. After this time, cells were disrupted to prepare the extracts for RNR as detailed in the Materials and methods section. Results are presented as means $\pm$ S.E.M. for three separate experiments, performed in duplicate.

Condition	RNR activity
IL-3	0.161 $\pm$ 0.038
No IL-3	0.034 $\pm$ 0.012
IL-3 readdition	0.022 $\pm$ 0.002

50 % of the initial level after 3 h without IL-3. dATP loss after IL-3 withdrawal was very similar to that of dTTP and dGTP, except that it initially decreased more slowly (70 % of the control level after 3 h of growth factor deprivation) (Figure 1B). After 8 h without IL-3, dATP levels declined in parallel with dTTP and dGTP. However, in contrast with the changes observed in these dNTP pools, dCTP levels were not affected by cytokine deprivation, as dCTP remained elevated after IL-3 withdrawal. All these changes in dNTP levels took place before DNA fragmentation was observed (Figure 1A). Therefore, in a similar manner to the situation found after treatment of the cells, in the presence of IL-3, with inhibitors of nucleotide metabolism, an early imbalance in the availability of the different dNTPs was induced in BAF3 cells by IL-3 deprivation.

Regulation of dNTP production by IL-3

We next examined the mechanism by which IL-3 regulates the short-term maintenance of dNTP levels. In the following experiments we measured the levels of dATP, dTTP and dGTP because dCTP levels were not affected by removal of IL-3. The experimental approach consisted of depriving cells of IL-3 for 6 h, then challenging them with the growth factor by readdition of IL-3 for 1 h, with or without 10 μ g/ml cycloheximide. The results presented in Table 1 show that the levels of dATP, dTTP and dGTP recovered after 1 h of restimulation with IL-3. Furthermore the presence of cycloheximide to inhibit protein synthesis did not prevent the recovery of dATP, dTTP or dGTP levels, suggesting that the effect of IL-3 on dNTP synthesis is due to the rapid stimulation of a pathway not requiring the synthesis of new proteins.

Figure 2 Effect of IL-3 on TK activity and on uridine incorporation

BAF3 cells were deprived of IL-3 (Wehi-3B-CM) for various times and TK activity was measured. (A) Cultures of BAF3 cells deprived of IL-3 for 6 h were restimulated with either Wehi-3B-CM or recombinant IL-3 and TK activity was determined. (B) BAF3 cells were deprived of IL-3 for 6 h and Wehi-3B-CM was added for 1 h. After this time the cultures were incubated with [5,6- 3 H]uridine as precursor (0.3 μ M, 8.3 μ Ci/ μ mol). The uptake of uridine was determined at different times as detailed in the Materials and methods section. Results are presented as means $\pm$ S.E.M. for three separate experiments, performed in duplicate. (A) TK activity: control with IL-3 (0.42 $\pm$ 0.05 nmol/h per mg of protein). Recombinant IL-3 was added for 1 h at 1 ng/ml. (B) [5,6- 3 H]uridine incorporation: ■, control with IL-3; ○, IL-3 deprivation for 6 h; ●, readdition of IL-3 (Wehi-3B-CM) for 1 h.

Effect of IL-3 on enzymes that control dNTP synthesis

The ability of the cell to replicate and repair DNA can be largely influenced by the availability of a balanced supply of dNTPs and hence by RNR activity. RNR activity varies greatly under different circumstances, including the cell cycle, and it is subjected to a complex allosteric and long-term regulatory mechanism [22]. Because recovery of nucleotide levels after IL-3 readdition takes place very rapidly and does not require protein synthesis, we then analysed the short-term modulation of enzymes involved in dNTP synthesis. IL-3 removal resulted in a complete loss of RNR activity but readdition of IL-3 (Wehi-3B-CM) for 1 h to deprived cells had no effect on the level of RNR activity (Table 2). However, in the long term (15 h after readdition of IL-3), RNR activity returned to the levels in cells maintained with IL-3 (results not shown). These results led us to suggest that synthesis of dNTPs from their precursors, such as uridine and thymidine for dTTP, through the salvage pathway, might be regulated by IL-3. This pathway involves a non-specific permease of the cell membrane and the action of TK to incorporate thymidine and uridine (reviewed in [23]). BAF3 cells were subjected to IL-3 deprivation and readdition as described above, and TK activity and [3 H]uridine incorporation into acid-soluble pools were determined. Results shown in Figure 2(A) indicate

Table 3 Effect of protein kinase inhibitors and the phorbol ester PDBu on TK activity

BAF3 cells deprived of IL-3 for 1 h were incubated for 30 min with the protein kinase inhibitors staurosporin (50 nM) or genistein (30 μ g/ml), before readdition of recombinant IL-3 for 30 min. Herbimycin (1 μ g/ml) was added to the cells for 16 h, after which they were either deprived of IL-3 for 1 h or deprived and restimulated with the recombinant cytokine, always in the presence of the inhibitor. The PKC activator PDBu (20 nM) was added to deprived cells for 30 min. Results are the means $\pm$ S.E.M. for three experiments performed in duplicate.

Condition	TK activity (nmol/h per mg of protein)
No IL-3	0.19 $\pm$ 0.02
IL-3 readdition	0.38 $\pm$ 0.34
+ Staurosporin	0.19 $\pm$ 0.02
+ Genistein	0.34 $\pm$ 0.04
+ Herbimycin	0.42 $\pm$ 0.01
PDBu	0.35 $\pm$ 0.04

that the loss of TK activity is an early event after IL-3 withdrawal because 1 h after cytokine removal there is a significant decrease in this activity. Figures 2(A) and 2(B) show that IL-3 deprivation for 6 h resulted in a marked decrease in TK activity and precursor incorporation compared with cells maintained in the presence of IL-3. Readdition of IL-3 for 1 h (either Wehi-3B-CM or recombinant IL-3) completely restored both parameters (Figures 2A and 2B) and this effect closely correlated with the recovery of dNTP pools observed in Table 1, suggesting that TK activity is involved in replenishing dNTP pools.

To understand the maintenance by IL-3 of dNTP pool balance in haemopoietic cells we determined the mechanism by which IL-3 upregulates TK activity. IL-3 receptor signalling includes activation of PKC [8] and tyrosine and serine phosphorylation of several substrates [9] to promote cell survival, proliferation and differentiation. To elucidate the mechanism by which IL-3 stimulates TK activity, the effect of inhibitors of different protein kinases was examined (see caption to Table 3). The concentrations used for the different inhibitors and for phorbol 12,13-dibutyrate (PDBu) have been previously shown to be able to inhibit and activate respectively these pathways in IL-3-dependent haemopoietic cells [24,25]. Staurosporin treatment completely abolished TK activation after readdition of recombinant IL-3, whereas genistein or herbimycin had no effect (Table 3). At the same time, the PKC activator PDBu replaced IL-3 for TK activation (Table 3), suggesting that PKC could be involved in signalling the cytokine effect on TK.

DISCUSSION

Pharmacological perturbation of dNTP pools has been previously shown to trigger DNA fragmentation and cell death in thymocytes [13], BAF3 cells [14], mammary tumour cells FM3A [15] and leukaemia cells [26]. Here we show that perturbation of the normal balance of dNTP pools is an early event in haemopoietic cells induced to undergo apoptosis after IL-3 removal. As early as 3 h after IL-3 withdrawal there are important deviations from the normal balance of dNTP seen in cells growing in the presence of IL-3. This imbalance is not a secondary effect of the apoptotic process because readdition of IL-3, 8–10 h after its removal, completely rescued cells from apoptosis [7]. Although the pattern of dNTP imbalance seen after IL-3 removal differs from that induced by common inhibitors of dNTP synthesis [14,15,26,27], the final outcome of these changes is a differential availability of dNTP for DNA synthesis and repair. Another

antitumour agent, 1-[1,3-dihydroxy-2-propoxymethyl]-5,6-tetramethylene uracil, specifically decreases the levels of dTTP, dATP and dGTP in hamster hepatoma cells [28] similar to our finding after the removal of IL-3 in haemopoietic cells. The reason for the maintenance of dCTP pool size after IL-3 withdrawal remains to be determined. In mutant fibroblasts lacking dCMP deaminase there is a decreased dTTP pool whereas dCTP accumulates [29]; there could therefore be a decrease in deaminase activity in IL-3-deprived cells. In contrast with the BAF3 cell model, where the cells are either growing or undergoing apoptosis, there are situations where quiescence is the alternative to growth, such as temperature shifting of thermolabile CHO mutant cells [30] or activation of human peripheral blood lymphocytes by phytohaemagglutinin [31,32]; in these cases the resulting perturbations in dNTP levels show a co-ordinated change with no imbalance of the DNA precursors. dNTP pool balance has been shown to be essential for fidelity in DNA synthesis in dividing cells (reviewed in [33]), and inhibition of DNA synthesis is sufficient to cause cell death [15]. In contrast, as IL-3 withdrawal does not result in complete arrest of the cell cycle in the short term [7], DNA synthesis could be occurring under conditions involving imbalanced dNTP pools. This imbalance in dNTP, responsible for misincorporation of nucleotides during DNA synthesis and repair [34,35], might therefore serve as a signal to initiate a death programme to prevent the appearance of genetic variants with chromosomal abnormalities. This mechanism would operate in the S phase of the cell cycle, during DNA replication and repair, and also during the G₁ and G₂ phases where DNA repair is active [36]. In support of a role for dNTP imbalance in the initiation of apoptosis, antineoplastic drugs affecting dNTP synthesis caused accumulation of p53 in the G₁/S phase transition leading to a p53-associated apoptotic response, to prevent genome instability [37].

In addition to the *de novo* pathway of dNTP synthesis, most cells have a salvage pathway by which dNTPs are synthesized from the products of nucleotide degradation present in the cell and the extracellular medium. There is a single permease with broad specificity allowing the influx and efflux of nucleosides, and the phosphorylation is performed by kinases present in the mitochondrial and the cytosolic compartments. In particular, cells of the haemopoietic system contain high levels of kinase activities for deoxyribonucleosides, although purine kinases are found at lower levels [23,38]. Among the cytosolic enzymes, TK is the most extensively studied. Its substrates are thymidine and uridine and it is inhibited by dTTP [39]. After IL-3 removal there are marked decreases in dTTP levels, TK activity and precursor uptake. Readdition of the cytokine to IL-3-deprived cells results in the rapid recovery of these parameters. It has been reported that TK is differentially phosphorylated in serine residues in HL60 and HeLa cells during the cell cycle [21,40] and the extent of phosphorylation gradually increased during growth stimulation, accompanied by a moderate increase in TK activity. In our cells, the occurrence of this post-translational modification is supported by the inhibition of IL-3-dependent TK activation by protein kinase inhibitors, such as staurosporin, as in G₂/M-arrested HeLa cells [40]. Although staurosporin has a variety of protein kinases as cellular targets [41], the stimulatory effect of PDBu, which specifically activates PKC, on TK activity, and the involvement of PKC in the early signals linked to the IL-3 receptor [8], suggest the involvement of this kinase in the effect of IL-3 on the activation of TK. This observation is in keeping with the finding that phorbol esters could in part replace the requirement for IL-3 in haemopoietic cells [8]. Tyrosine phosphorylation is also known to mediate IL-3 receptor signalling [9,11]. However, inhibitors of tyrosine kinases such

genistein and herbimycin do not block the effect of IL-3 on TK activation, indicating that tyrosine phosphorylation is not involved in this pathway.

IL-3 affects rate-limiting steps of the salvage synthesis of dNTP as well as altering RNR activity, and this action may have a role in the regulation of apoptosis by IL-3, perhaps acting in a concerted manner with the *de novo* pathway of dNTP synthesis. Cells lacking TK activity have been shown to have a lower rate of DNA synthesis and a longer generation time [42]. Furthermore, deregulated expression of TK activity has been reported to be correlated with tumorigenicity [43] and cell resistance to radiation [44]. Our results raise the hypothesis that regulation of TK by growth factors could be a check point to signal progression through the cell cycle or diversion to apoptosis. Experiments are currently being carried out to define the role of TK in the regulation of apoptosis in haemopoietic cells and the mechanism of dNTP imbalance-induced apoptosis in these cells.

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