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dNTP pools imbalance as a signal to initiate apoptosis

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Abstract. Fidelity in DNA synthesis and repair is largely dependent on a balanced supply of deoxynucleotide triphosphate (dNTP) pools. Results from different groups have shown that alterations in dNTP supply result in DNA fragmentation and cell death with characteristics of apoptosis. We have recently shown that in apoptosis driven by deprivation of interleukin-3 (IL-3) in a murine hemopoietic cell line, there is a rapid imbalance in the availability of dNTP that precedes DNA fragmentation. In these cells, dNTP pool balance is closely coupled to the function of the salvage pathway of dNTP synthesis. Apoptosis, induced by treatment of these cells with drugs that inhibit the de novo dNTP synthesis, is prevented when dNTP precursors are supplied through the salvage pathway. IL-3 regulates thymidine kinase activity, suggesting that alterations in dNTP metabolism after IL-3 deprivation could be a relevant event in the commitment of hemopoietic cells to apoptosis.
Key words. Apoptosis; dNTP metabolism; thymidine kinase; interleukin-3; blc-2; hemopoietic cells.

Introduction

Cell population dynamics depend upon changes in the balance between cell proliferation and death. Transformed cells may be those which either proliferate in the absence of growth factors or fail to undergo apoptosis upon factor removal [1]. Tumour cells are susceptible to apoptosis, and certain therapeutic strategies have been developed to induce cell loss by apoptosis. Indeed, a number of antineoplastic drugs and treatments exert their cytotoxic effect by inducing apoptosis [2], and the drug and radiation resistance of many tumours could be ascribed to the failure of certain cancer cells to commit apoptosis due to mutations in p53 antioncogene or deregulation of *bcl-2* expression [1, 3]. The maintenance of balanced deoxynucleotide triphosphate (dNTP) pools is critical for DNA replication and repair; it is mainly achieved by regulation of the activity of enzymes of the de novo pathway of synthesis of dNTPs [4]. Whereas moderate perturbation of dNTP pools affects genetic stability of cells [5], with the appearance of frequent mutations [6] and strand breaks [7], severe imbalance of dNTP pools causes cell death [8]. Several antineoplastic agents which inhibit DNA precursor synthesis have been reported to kill lymphoid cells by induction of apoptosis [3], and inherited deficiencies in enzymes such as adenosine deaminase and purine nucleoside phosphorylase, which produce imbalanced accumulation of dNTPs, result in lymphoid cell death [9]. Results from our laboratory have shown that inhibition of thymidylate synthase (an enzyme of the de novo synthesis of dNTP) with 5-fluoro-2′-deoxyuridine, which perturbs deoxyribonucleotide metabolism, drives the IL-3-dependent cell line BAF-3 to enter apoptosis even in the presence of IL-3 [10], and removal of IL-3 leads to an early imbalance in dNTP pools during the apoptotic process [11].

In the present review we summarize our current knowledge of the role of perturbations in DNA precursor levels and dNTP metabolism in signalling the commitment of mammalian cells to apoptosis.

DNA precursor synthesis in eukaryotes: an overview

There are basically two routes to synthesis of dNTPs in eukaryotes (fig. 1). Ribonucleotide diphosphates are formed first and subsequently reduced to deoxyribonucleotides by ribonucleotide reductase (RNR). Through the action of this enzyme, three deoxyribonucleotides, deoxyadenosine diphosphate (dADP), deoxycytidine diphosphate (dCDP) and deoxyguanosine diphosphate (dGDP), are obtained directly, and after phosphorylation by nucleoside diphosphate kinases, the respective dNTPs are formed. These dNTPs are now ready for DNA synthesis and repair. The DNA precursor dTTP is also formed through the reductase, but requires a more complex transformation (fig. 1). In addition to this de novo road, different kinases catalyse the phosphorylation of deoxyribonucleosides, allowing the reutilization of material obtained from degradation of DNA. This is called the salvage pathway, and it regulates the flux of deoxyribonucleosides in and out of cells and, together with the de novo pathway, contributes to the setting of the intracellular levels of dNTPs. Two enzymes of the de novo pathway are subject to allosteric control and, therefore, targets for regulatory signals:

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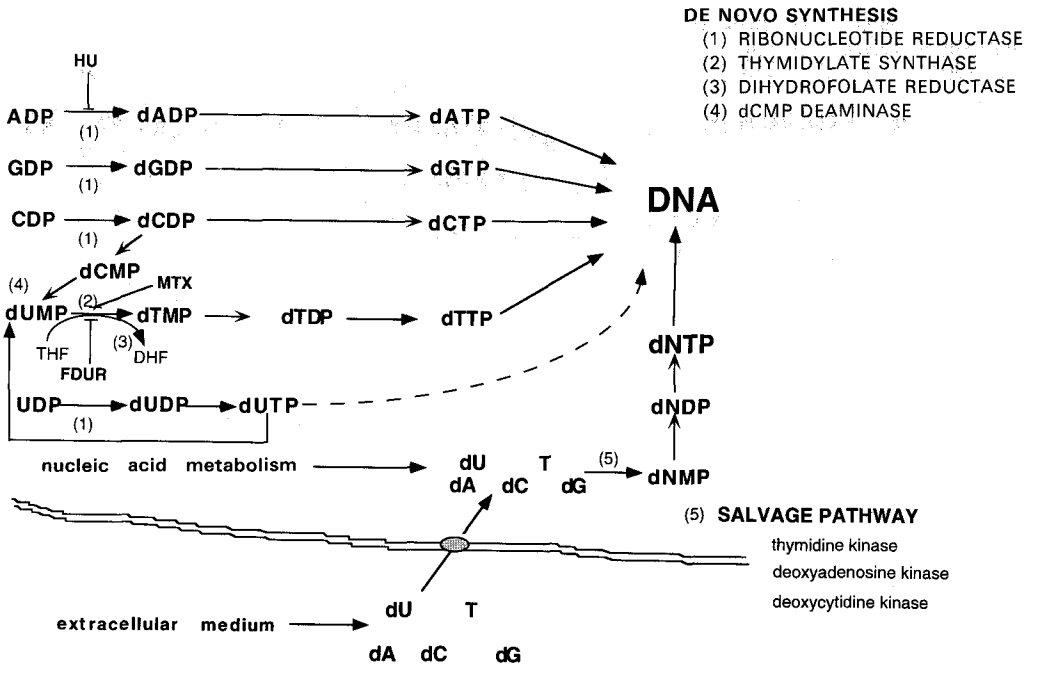


Figure 1. Pathways of dNTP synthesis in eukaryotes.

RNR and deoxycytidine 5′-monophosphate (dCMP) deaminase.

RNR activity largely controls the overall de novo pathway. This enzyme has two subunits, R1 and R2. The regulatory subunit, R1, is subject to a complex allosteric regulation responsible for maintaining balanced nucleotide pools, and the R2 subunit is phosphorylated in vivo by $p34^{cdc2}$ and CDK2 [12]. Reductase activity is also modulated at the transcriptional level during the cell cycle [12]. The existence of a tyrosyl free radical in the small subunit of the reductase complexed with Fe^{2+} is essential for enzyme activity, and hydroxyurea and related drugs inactivate the enzyme by scavenging the tyrosyl free radical (fig. 1).

dCMP deaminase is the second enzyme of the de novo pathway subject to allosteric control. Deoxycytidine 5′-triphosphate (dCTP) activates and dTTP inhibits the enzyme allosterically from all sources. In mammalian cells in culture, dCMP deaminase provides most of the deoxyuridine monophosphate (dUMP) required for dTTP synthesis when deoxyuridine and thymidine are absent from the medium. Thymidylate synthase links dNTP synthesis to folate metabolism and is dependent on an active dihydrofolate reductase. These two enzymes lack allosteric control and normally have no rate-limiting function. They are the targets for fluorouracil derivatives and methotrexate, respectively, which are commonly used in chemotherapy.

Mammalian cells contain two sets of salvage kinases: one type is present in the cytosol, the other in the mitochondria. One of these enzymes, thymidine kinase, accepts deoxyuridine and deoxythymidine as substrates and is inhibited by dTTP [4]. Thymidine kinase from herpes simplex virus-1 (HSV-1) has a wider variety of substrates, and this property has been exploited in the use of suicide substrates, such as gancyclovir, in gene therapy [13]. It has been reported that TK polypeptide is regulated by phosphorylation in serine residues in HL60 and HeLa cells during the cell cycle [14], and the extent of phosphorylation gradually increases during growth stimulation. A different cytosolic kinase primarily phosphorylates deoxycytidine, but deoxyadenosine and deoxyguanosine could also be substrates. Two separate mitochondrial kinases have been described for thymidine/deoxycytidine or deoxyguanosine [15].

Many of the enzymes of dNTP synthesis, both those catalysing the de novo pathway and those participating in the salvage of deoxynucleotides, increase in activity when cells prepare for DNA synthesis. This regulation takes place at the transcriptional level. Inactivation by phosphorylation of the antioncogenic protein Rb stimulates the transcription factor E2F1, which in turn transactivates a number of genes involved in the G1/S transition of the cell cycle and in DNA synthesis. Among these genes RNR, thymidylate synthase, dihydrofolate reductase and thymidine kinase are frequently observed to be activated at the transcriptional level [16]. The fine tuning of all these regulatory mechanisms is needed in order to maintain the fidelity of DNA synthesis and repair processes.

dNTP supply, mutation rate and carcinogenesis

The degree of error in DNA replication and repair contributes to the characteristic mutation rate of any organism and influences the equilibrium of species preservation and evolution; in higher eukaryotes it may

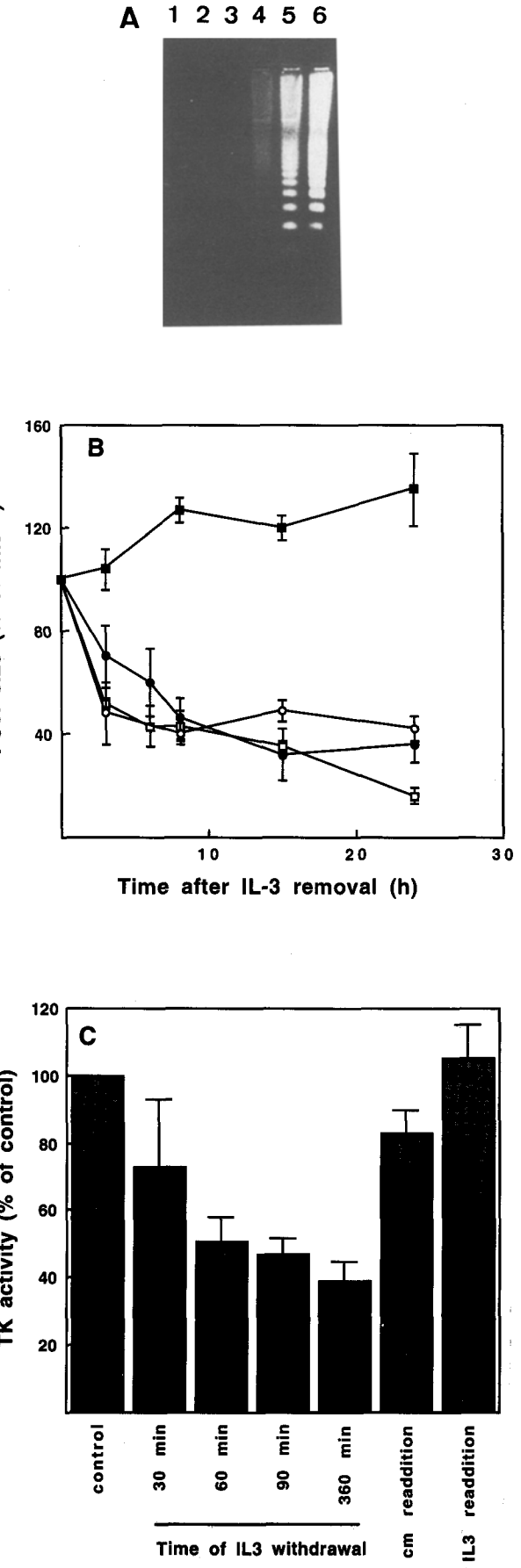
also contribute to cell transformation and tumorigenesis. Different studies using natural templates of known nucleotide sequence have stressed the importance of relative dNTP concentrations in mediating base substitutions and misincorporation [17]. The cell requires a balanced supply of each of the four dNTPs to replicate and repair its DNA properly, and perturbations in dNTP pools have consequences for the genetic stability of the cell.

A multienzyme complex for metabolic channelling of dNTPs during DNA replication, replitase, was proposed in the previous decade [18]. This complex produces dNTPs and delivers them to DNA polymerase activity, which also resides in the complex. Structural interactions within this complex form the basis of internal control to keep these key biosynthetic processes efficient and in balance. The active complex is formed in the nuclear region during the S phase of the cell cycle, when DNA is being replicated. However, later work has cast serious doubts on the replitase concept, and there is at present little evidence for it in mammalian cells. Transient imbalance in DNA precursors induced by treatment with nucleosides increases the mutation frequency at different loci [19], and chronic imbalance in dNTP pools caused by genetic alterations in enzymes controlling the de novo synthesis of dNTPs correlates with increased spontaneous mutation rates at several genetic loci [17, 20]. The types of mutations induced in mammalian cells by specific imbalances of DNA precursors have been studied at two selectable loci, *aprt* and *hprt*, by sequencing the coding sequences of mutant clones (reviewed in ref. 21). The molecular analysis revealed that the mutations were predominantly base substitutions involving misincorporations of the nucleotide in excess [22, 23]. More recently, Daré et al. using dCMP deaminase-deficient cells, have shown that the effects of dNTP pool imbalance on the fidelity of DNA replication require large pool changes and specific ratios between the different precursors [20]. This is supported by the observation that specific dNTP pool depletions, rather than general imbalance of pools, give rise to the inhibition of DNA excision repair in human fibroblasts [24]. Perturbations in dNTP pools may also have a role during oncogenic transformation. Bone marrow cells from patients with megaloblastic anaemia display a marked imbalance of the four dNTPs with excessive accumulation of dCTP [25]. On the other hand, in vitro experiments have shown that thymidylate excess and deprivation are recombinogenic and provoke chromosome rearrangements with oncogenic transformations [26].

dNTP imbalance and cell death

Perturbation of dNTP pools has been suggested to trigger DNA fragmentation and cell death in different cell types. Early studies showed deletion of T lymphocytes associated with adenosine deaminase and purine nucleoside phosphorylase deficiencies; this cell death was preceded by the appearance of DNA laddering [27]. 'Thymineless death' is found when severe dTTP depletion takes place following drug treatment or mutations. In mouse FM3A cells lacking thymidylate synthase activity, dTTP decrease is accompanied by depletion of deoxyguanosine triphosphate (dGTP) and an increase in deoxyadenosine triphosphate (dATP) and deoxyuridine triphosphate (dUTP) levels, resulting in single and double DNA strand breaks. Studies from Yoshioka and collaborators suggested that dNTP imbalance would result in the activation of the gene coding for an endonuclease, causing DNA double-strand breaks and cell death [28]. Other authors postulated that a rise in the dATP pool would inhibit DNA repair of single-stranded DNA breaks in resting lymphocytes by blocking ribonucleotide reductase or DNA ligation [29], leading eventually to cell death. According to a different proposed mechanism, the accumulation of dUTP and lack of dTTP lead to extensive incorporation of uracil into DNA, followed by massive excision-repair and fragmentation of DNA [30]. In support of this model, Canman et al. have shown that overexpression of dUTPase, involved in depleting dUTP levels to avoid uracil incorporation into DNA, renders human tumour cells resistant to fluorodeoxyuridine cytotoxicity [31]. The relevance of dNTP alterations in cell death processes and the regulatory role of the salvage pathway of dNTP synthesis has been demonstrated in experiments where addition of deoxynucleoside precursors to the culture media blocked cell death induced by methotrexate or hydroxyurea (ref. 32; Oliver and López-Rivas, unpublished results). A similar role of the salvage pathway has been demonstrated in erythroblasts from mice with experimental folate deficiency anemia, where addition of thymidine is sufficient to protect cells from apoptosis [33].

Cell death induced by dNTP imbalance has been associated with common pathways leading to apoptosis. Results from our group [10] and others [34] have shown that overexpression of the antiapoptotic gene Bcl-2 protects cells from apoptosis induced by inhibitors of dNTP de novo synthesis. In order to define the pathway by which Bcl-2 protein blocks programmed cell death, we determined the variations in dATP and dTTP pools in cells overproducing Bcl-2 protein after fluorodeoxyuridine (FDUR) treatment. We did not find any substantial variation in the levels of dNTP between both types of cells that could account for the difference in their response to FDUR (fig. 2). This supports the hypothesis that Bcl-2 has an effect in a distal common pathway of cell death that can be activated by different signals. Thus, although perturbations in dNTP pools are necessary to induce cell death in the parental cell line, activation of a downstream pathway is needed in order for the cell to enter



the apoptotic process. Moreover, accumulation of the antioncogenic activity p53 follows dNTP imbalance after treatment with the appropriate drugs, and inactivation of p53 results in the loss of sensitivity to antineoplastic drugs affecting the synthesis of DNA precursors [35].

Results from our laboratory have also implicated dNTP pool perturbations in apoptosis driven by withdrawal of the hemopoietic cytokine IL-3 [11]. Cytokine deprivation in primary cells or cell lines derived from bone marrow leads to a program of events typical of apoptosis, including chromatin digestion into oligonucleosome-size fragments (fig. 3A; refs 36 and 37). Activation of an endogenous endonuclease has been proposed to be necessary for apoptosis, and the apoptotic process becomes irreversible at a time when DNA fragmentation is first observed [37]. In hemopoietic BAF3 cells, IL-3 withdrawal leads to a rapid decrease in the size of dATP, dTTP and dGTP pools without affecting dCTP levels (ref. 11; fig. 3B). This imbalance in dNTP pools precedes DNA fragmentation and is accompained by downregulation of enzymes that control the de novo and salvage pathways of dNTP synthesis, ribonucleotide reductase and thymidine kinase, respectively. Readdition of IL-3 results in a rapid, protein synthesis-independent restoration of normal dNTP pools and enhanced thymidine kinase (TK) activity (fig. 3C; ref. 11). Upregulation of thymidine kinase activity after IL-3 readdition is prevented by the protein kinase C (PKC) inhibitor staurosporin, but not by tyrosine kinase inhibitors [11]. 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 [11]. 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 are relevant in the commitment of hemopoietic cells to apoptosis. Thymidine kinase has been used as a malignancy marker for a variety of tumours, and there is a close correlation between thymidine kinase activity of

Figure 2. Effect of IL-3 withdrawal on DNA fragmentation, dNTP pool size and thymidine kinase activity. In panel *A*, DNA was extracted and subjected to agarose gel electrophoresis. Lanes 1, 2, 3, 4, 5 and 6 correspond to 0, 3, 5, 8 15 and 24 h after IL-3 deprivation. At these times the levels of the different dNTPs were determined in parallel cultures (panel *B*). Results are the percent of the initial levels of the different dNTPs and represent the average $\pm$SE of two separate experiments in duplicate for dCTP (51.0 ± 4.8 pmol/10^6 cells) (■) and dGTP (4.8 ± 0.5 pmol/10^6 cells) (○) and four separate experiments for dATP (6.2 ± 0.6 pmol/10^6 cells) (●) and dTTP (22.2 ± 1.7 pmol/10^6 cells) (□). Panel *C* represents the effect of IL-3 on thymidine kinase activity. BAF3 cells were deprived of IL-3 for various times, and TK activity was measured. 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. TK activity: control with IL-3 (0.42 ± 0.05 nmol/mg protein/h). Recombinant IL-3 was added at 1 ng/ml for 1 h.

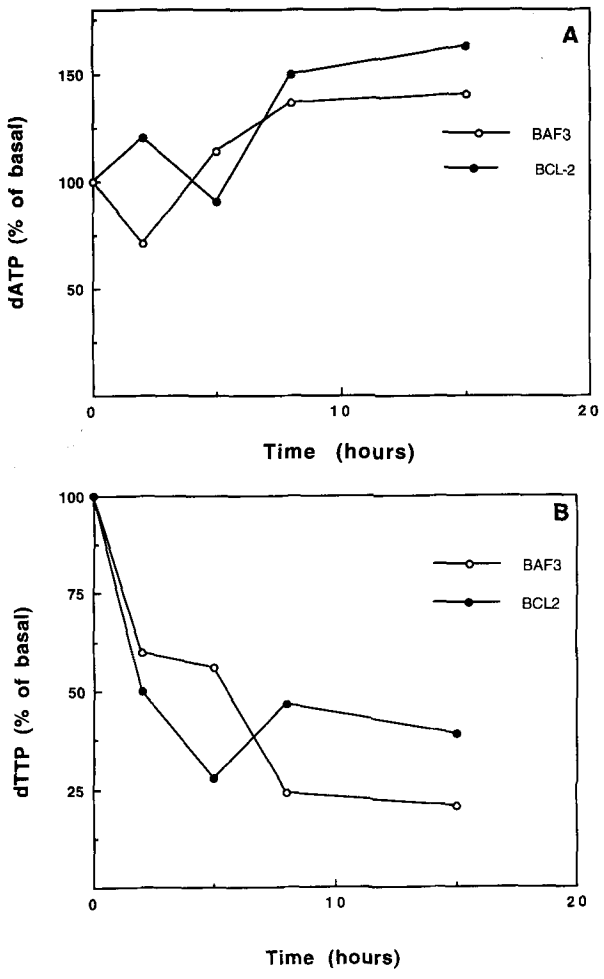


Figure 3. dATP and dTTP pool variations in BAF3 cells after 5 μM FDUR treatment. Basal levels for dATP were 5.5 ± 0.5 and 5.9 ± 10.3 pmol 10^6 cells for BAF3 and $BAF3^{bcl\text{-}2}$ cells, respectively. For dTTP the values were 20.0 ± 1.5 and 15 ± 2.2 pmol/10^6 cells in BAF3 and $BAF3^{bcl\text{-}2}$ cells, respectively. These values are from at least three separate experiments.

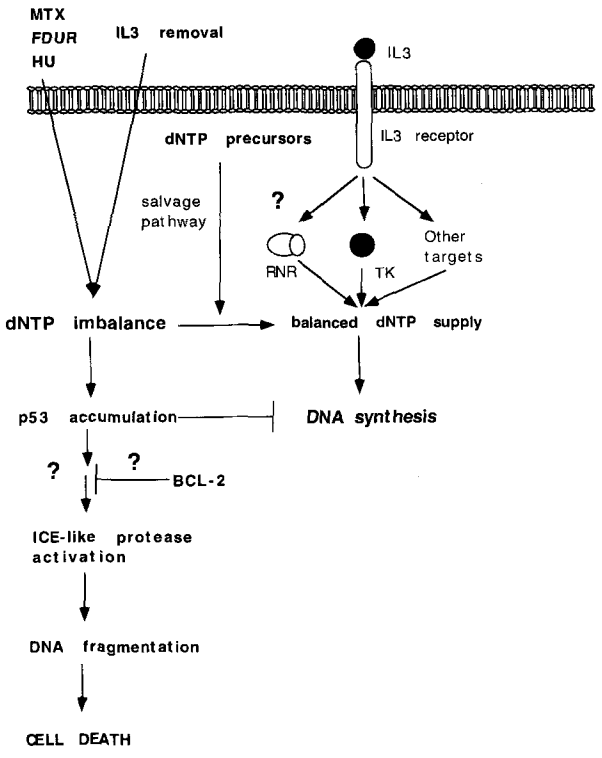


Figure 4. A model for the involvement of dNTP pool imbalance in the apoptotic pathway.

tumour cells and resistance to therapeutic agents [38, 39]. Enhanced thymidine kinase activity may influence the ability of the cell to maintain an adequate dNTP supply for DNA synthesis in situations where the dNTP de novo pathway is limited by drugs, and this could explain the resistance of certain tumour cells to chemotherapy.

Conclusions

As summarized in figure 4, we propose a model in which pertubations in dNTP levels are an early signal for the cell to enter the apoptotic pathway in cells treated with antimetabolite drugs or after removal of cytokines from dependent cells. Restoring dNTP levels by addition of appropriate precursors results in the prevention of apoptosis in drug-treated cells. We are currently trying to analyse this further by maintaining dNTP levels in hemopoietic cells using an approach based on the overexpression of enzymes involved in dNTP synthesis. In this regard, stable expression of a heterologous thymidine kinase gene, the HSV-1 TK, renders the cells resistant to apoptosis induced by IL-3 removal and drug treatment (Oliver and López-Rivas, unpublished results). However, the downstream events after dNTP imbalance needed for the cell to reach the stage of commitment to complete apoptosis are not known. Cells overproducing Bcl-2 protein will not undergo apoptosis in spite of the imbalance in intracellular dNTP pools. Under these conditions, accuracy in DNA replication and repair will not be guaranteed, and therefore genetic variants lacking essential growth-controlling functions may appear, increasing the possibility of oncogenic transformation. The same scenario could be envisaged in cells with an inactive p53 tumour suppressor protein, which is the most frequently reported mutation associated with human tumours [40].

More work needs to be done to analyse how perturbations in DNA precursor levels induce the apoptotic programme and particularly to demonstrate the activation of the execution machinery involving interleukin-1β converting enzyme (ICE)-like [14] proteases.

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