Overexpression of a Heterologous Thymidine Kinase Delays Apoptosis Induced by Factor Deprivation and Inhibitors of Deoxynucleotide Metabolism

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Perturbing deoxynucleoboside triphosphate (dNTP) metabolism with inhibitors of the de novo synthesis of dNTP causes apoptosis in the interleukin-3 (IL-3)-dependent pre-B cell line BAF3. Under these conditions apoptosis is prevented when deoxynucleosides for dNTP synthesis are supplied in the culture medium. On the other hand, removal of IL-3 from cultures of BAF3 cells resulted in down-regulation of thymidine kinase activity, rapid imbalance in dNTP levels, and apoptosis. In this study we show that overexpression of a heterologous thymidine kinase, herpes simplex virus thymidine kinase (TK), in BAF3 cells protects these cells from apoptosis induced by either inhibitors of dNTP synthesis or IL-3 deprivation. This protection against apoptosis is abrogated by 9-(4-hydroxybutyl)-N²-phenylguanine, a specific inhibitor of herpes simplex virus-1 TK. These results suggest that deoxynucleoboside kinases, particularly TK, may be important in the regulation of apoptosis in hemopoietic cells.

Cell population dynamics depend upon changes in the balance between cell proliferation and death. Transformed cells may be those that either proliferate in the absence of growth factors or fail to undergo apoptosis upon factor removal (1). Tumor cells are susceptible to apoptosis, and certain therapeutic strategies for cancer have been developed to induce apoptosis. Indeed, a number of antineoplastic drugs and treatments exert their cytotoxic effect by inducing apoptosis (2). Furthermore, the drug and radiation resistance of many tumors can be ascribed to the failure of cancer cells to enter apoptosis due, for example, to mutations in p53 or deregulation of the expression of proteins of the bcl-2 family (1, 3).

The maintenance of balanced dNTP pools is critical for DNA replication and repair; under normal culture conditions, 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 that 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). Previous 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 deoxynucleoboside metabolism, drives the IL-3-dependent cell line BAF3 to enter apoptosis even in the presence of IL-3 (10). Furthermore, removal of IL-3 from BAF3 cell cultures leads to an early imbalance in dNTP pools during apoptosis (11).

In addition to the de novo pathway for dNTP synthesis, mammalian cells, in particular those of the immune system, contain kinase activities for deoxynucleobosides (4). These salvage enzymes function in the reutilization of degradation products from nucleic acids or precursors from the extracellular medium. Thymidine kinase is perhaps the best characterized of these enzymes; its substrates are thymidine and deoxyuridine, and it is inhibited by dTTP (4). It has been used as a malignancy marker for a variety of tumors (12, 13), and tumor cells deficient in this enzyme show a lower oncogenic potential than the wild-type cells from which they derive (14). Moreover, a recent report has also demonstrated that TK is a major radioresponse determinant in rat glioma cells (15).

It has been suggested that this reutilization pathway, acting in a concerted manner with other salvage enzymes and the de novo pathway, could also regulate intracellular levels of dNTPs (4) and protect cells from apoptosis (16–18). Here, we show that the potentiation of this salvage pathway either by the supply of dNTP precursors or by overexpression of a heterologous TK (HSV-1 TK) delays programmed cell death induced by IL-3 deprivation and drugs that inhibit dNTP metabolism in BAF3 cells.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium and fetal bovine serum were obtained from Life Technologies, Inc. Methotrexate was from Cyanamid Iberica, Division Lederle. Deoxy[8-3H]ATP (24 Ci/mmol), deoxy[8-3H]GTP (16.9 Ci/mmol), deoxy[5-3H]CTP (18 Ci/mmol), and [methyl-3H]thymidine (49 Ci/mmol) were from Amersham. [methyl-3H]thymidine (49 Ci/mmol) was purchased from ICN Biochemicals. Synthetic DNA templates, 5-fluoro-2'-deoxyuridine, hydroxyurea, and other reagents of the purest grade available were obtained from Sigma and Boehringer Mannheim. The HSV-1 TK inhibitor 9-(4-hydroxybutyl)-N²-phenylguanine (HBPG) was kindly provided by Dr. George E. Wright (University of Massachusetts Medical School, Worcester, MA).

Cell Cultures and Transfection Experiments—Murine IL-3-depend-
ent BAF3 cells (19) were maintained in RPMI medium containing 10% fetal bovine serum, 1 mM glutamine, and 10% conditioned medium from the IL-3-producing cell line Wehi-3B. For transient expression experiments, BAF3 cells were transfected with HSV1-TK cDNA cloned into MFG-S plasmid (Somatix) or with a control cDNA (the neomycin-resistance gene, neo) by electroporation of 10–20 μg of DNA at 350 V or alternatively by lipofection with 5 μg of Lipofectin (Life Technologies, Inc.) and 2.5 μg of DNA. Cells were cultured in the presence of IL-3 for 24–48 h, and thymidine kinase activity was measured. For stable expression of HSV thymidine kinase activity, cells were cotransfected by electroporation with the previous TK cDNA and a plasmid conferring resistance to puromycin, pBabe Puro (20). Puromycin was added at 4 μg/ml to the cultures to select cells expressing resistance to this marker.

Analysis of DNA fragmentation and cell cycle were performed according to published procedures (21).

Deoxyribonucleoside Triphosphate Pool Assay—Preparation of cell extracts was essentially as described (22). dNTPs were determined by the DNA polymerase assay (23) using a synthetic DNA template and the Klenow fragment of DNA polymerase. The intracellular concentrations of dNTP were estimated from calibration curves obtained using pure standards.

Thymidine Kinase Activity—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, and 1 mM phenylmethylsulfonyl fluoride, subjected to Dounce homogenization, and sonicated to completely disrupt the cells. The extract was cleared of cellular debris.

**FIG. 1. Addition of deoxyribonucleosides to the culture medium can regulate apoptosis in BAF3 cells.** BAF3 cells cultured in the presence of IL-3 were incubated with 5 μM MTX or 1 mM HU. At different times after drug addition, cell viability was determined (A). Results are the average of three separate experiments. DNA was isolated from cells treated for 0, 5, 8, and 15 h with 5 μM MTX or 1 mM HU and subjected to agarose gel electrophoresis (B). C, BAF3 cells growing in the presence of IL-3 were incubated with 5 μM methotrexate (MTX), 5 μM methotrexate + 50 μM thymidine (MTX + T), 50 μM hydroxyurea (HU), or 50 μM hydroxyurea + 100 μM deoxyguanosine/1 μM deoxyadenosine (HU + AG) or with no addition (control) and incubated for 15 h. Cells were fixed, treated with propidium iodide, and subjected to fluorescence-activated cell sorter analysis as described previously (18).

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by centrifugation, and the remaining supernatant containing a minimum of 2 mg of protein/ml was assayed for TK activity essentially as described (24).

Statistical Analysis—The statistical significance of the data was determined after applying Student’s t test.

RESULTS AND DISCUSSION

Deoxynucleosides Prevent the Induction of Apoptosis by Inhibitors of dNTP Synthesis in Hemopoietic BAF3 Cells—Dihydrofolate reductase and ribonucleotide reductase catalyze key steps in the de novo production of dNTPs. Inhibitors of these enzymes, such as methotrexate (MTX) and hydroxyurea (HU), are commonly used in antineoplastic treatment, and they also induce apoptosis in hemopoietic BAF3 cells even in the presence of IL-3 (10). After 8 h of treatment with either drug, loss of cell viability, as assessed by cell membrane integrity, was not observed (Fig. 1A); however, at this time DNA was digested into oligonucleosome-sized fragments (Fig. 1B). Cell death could be clearly observed after 15 h of treatment with either inhibitor. Addition of deoxynucleosides to the medium facilitates dNTP synthesis; these precursors are transported by a nonspecific permease across the cell membrane and modified by the action of deoxynucleoside kinases to generate dNTP pools (4), thus bypassing the de novo synthesis pathway. The presence of 50 μM thymidine in the culture medium completely prevented the appearance of the sub-G1 peak of apoptotic cells in cultures of BAF3 cells treated with MTX for 15 h (Fig. 1C) and inhibited DNA fragmentation and cell death (data not shown). Inhibition of HU-induced apoptosis was achieved by incubating the cells with 1 μM deoxyxadenosine and 100 μM deoxynucleosine (Fig. 1C). This precursor combination was used in the study of Lagergren and Reichard (25), in which it reversed the inhibition of DNA synthesis by HU. These data suggest that salvage enzymes involved in the phosphorylation of these precursors are probably important in the maintenance of dNTP balance and the inhibition of cell death (17, 18). A similar role of this salvage pathway has been demonstrated in erythroblasts from mice with experimental folate deficiency anemia, in which the addition of thymidine is sufficient to protect cells from apoptosis (16).

Overexpression of HSV-1 Thymidine Kinase Suppresses Apoptosis Induced by IL-3 Removal and Inhibitors of dNTP Synthesis—To determine the role of TK activity in the regulation of apoptosis, we have examined the effect of overexpressing heterologous HSV-1 TK (26) on the entry of cells into apoptosis after IL-3 withdrawal or drug treatment. Initially, we transiently expressed HSV-1 TK in BAF3 cells and determined both the level of TK activity and apoptosis in bulk-transfected cells. Bulk-transfected cell populations were deprived of IL-3 for 24 h, and viability was measured at this time. The results from eight independent transfection experiments are shown in Fig. 2A; a correlation was found between the level of expression of TK activity and protection from cell death. Whereas cell viability decreased to 20% in control (vector-transfected) cells, transfection of HSV-1 TK resulted in up to 80% viable cells after IL-3 deprivation. These trypan blue-excluding cells in the HSV-1 TK-transfected cultures were viable because they were able to grow in response to IL-3, with a generation time similar to that of cells not subjected to IL-3 withdrawal (data not shown).

We next determined whether stable HSV-1 TK expression could inhibit apoptosis. By transfecting BAF3 cells with the HSV-1 TK cDNA, several clones were generated that expressed high levels of TK activity. From these clones, clone TK2 was chosen because it did not release any soluble factor to the culture media to allow cell survival in the absence of IL-3, which would mask the effect of TK overexpression. Cell viability in a control clone expressing only resistance to puromycin (puro3) decreased to less than 30% after 24 h in the absence of IL-3, and there was a complete loss of cell viability after 48 h of cytokine deprivation (Fig. 2B). In contrast, viability of TK2 cells was maintained at values higher than 90 and 30% after 24 and 48 h of IL-3 withdrawal, respectively (Fig. 2B). Furthermore, the clone overexpressing HSV-1 TK was able to maintain TK activity after deprivation of IL-3, in contrast to what was observed in puro3 cells (data not shown) and parental BAF3 cells (11). We have also determined the effect of HSV-1 TK overexpression on the viability of cells treated with inhibitors of dNTP metabolism such as MTX and FdUrd, which rapidly deplete the cells of dTTP. Results in Fig. 3A and B show that cells overexpressing HSV-1 TK are more resistant to cell death induced by increasing doses of FdUrd or MTX.
We also measured intracellular dNTP levels after MTX treatment or IL-3 withdrawal in control and HSV-1 TK-transfected cells. The dNTP pools in untreated cultures from both cell types were not significantly different (Fig. 4, legend), but the cell response to an antimetabolite drug or IL-3 deprivation was markedly different (Fig. 4). Control cells treated for 3 h with 5 μM MTX exhibited a 66% decrease in dTTP and dGTP levels, whereas dATP and dCTP remained unchanged. In IL-3 deprivation experiments, the levels of dATP, dGTP, and dTTP after 8 h in the absence of IL-3 decreased to about 40–50% of the initial levels, whereas the dCTP level was only slightly changed, as reported previously (11). Interestingly, in cells overexpressing HSV-1 TK, treatment with MTX had no effect on the intracellular dATP, dGTP, and dTTP levels and had little effect on the dCTP pool (67% of the level found in untreated cells). Furthermore, removal of IL-3 from these cells did not induce a decrease in the values of all four dNTPs, showing a general maintenance of dNTP levels as a result of TK overexpression. Although one would have expected only the dTTP level to be maintained in HSV TK-overexpressing cells, allosteric activation of ribonucleotide reductase by dTTP might be responsible for the elevated pool of dGTP, which in turn can stimulate the reduction of ADP to dADP (27).

Earlier reports have shown that introduction of herpes virus DNA into TK-deficient cells was able per se to alter the sensitivity to β-interferon, irrespective of the acquisition of TK activity (28, 29). To rule out this possibility in our experiments, we took advantage of a recently characterized specific inhibitor of HSV-1 TK (30). This compound, HBPG, did not inhibit cellular TK activity as assayed in vitro on cell extracts from control cells and had no effect on [3H]thymidine incorporation in growing cells (data not shown). Fig. 5A shows that HBPG did not affect the cell viability of control cells in either the presence or absence of IL-3. However, in clones overexpressing HSV-1 TK, 10 μM HBPG produced a 67% inhibition of the TK activity present in cell extracts from these cells (data not shown). In
these cells, the inhibitor did not induce apoptosis in the presence of IL-3 (Fig. 5B). However, in IL-3-deprived TK-transfected cells, HBPG significantly reduced cell viability (Fig. 5B) and induced a sub-G₁ population of apoptotic cells (Fig. 5D) to values similar to those of control cells (Fig. 5, A and D). The inhibition of viral TK by HBPG also increased the sensitivity of TK2 cells to FdUrd and MTX as determined by measuring cell viability (Fig. 5C) or by the generation of sub-G₁ cells in cell cycle analysis experiments (Fig. 5D). In summary, these results indicated that the protection from apoptosis in cells overex-

**Fig. 5. Effect of HBPG on the suppression of apoptosis by HSV-1 TK overexpression.** The HSV-1 TK inhibitor HBPG was used at 10 μM final concentration. A and B, the effect of HBPG treatment on the suppression of cell death by HSV-1 TK overexpression after IL-3 removal. In C, results are shown for cell death induced after 15 h of treatment with 10 μM MTX or 10 μM FdUrd in the presence or absence of 10 μM HBPG. Data shown are the average ± S.D. of at least two independent experiments. D, analysis by flow cytometry of apoptosis induced under the experimental conditions presented in previous panels of this figure after 15 h of either IL-3 removal or MTX treatment.
pressing HSV-1 TK was the direct outcome of the presence of the viral TK activity and not a consequence of the introduction of viral DNA.

Whereas many intracellular signals are known to decline after removal of IL-3 from dependent cells (31–33), only overexpression of oncogenes such as myc (34) or bcl-2 (21), activators of the ras pathway (35, 36), or activated abl kinase (37) has previously been shown to modulate apoptosis. Our data demonstrate that a single enzyme involved in nucleotide metabolism can exert a similar effect, which suggests that regulation of the dNTP supply may be a control point in apoptosis.

Although mammalian TK expression is cell cycle-regulated, with peak gene expression occurring immediately before entry into S-phase (38), we have demonstrated that IL-3 also regulates TK activity posttranslationally (11). Serum has also been shown to regulate the phosphorylation state and activity of TK in HL-60 cells (24). The vector used in our transfection experiments constitutively expresses HSV TK throughout the cell cycle, and the viral TK does not seem to be down-regulated after withdrawal of IL-3, resulting in increased TK activity even in the absence of IL-3.

Under normal culture conditions, the viability and growth of cells do not seem to require the uptake of exogenous deoxyribonucleosides unless the endogenous synthesis of dNTP has been inhibited by treatment with drugs (17, 18). This is because most culture media contain high levels of folic acid, which allows the continued synthesis of tetrahydrofolate derivatives required for the synthesis of nucleotides and particularly of thymidylate. However, in culture media containing concentrations of folic acid comparable to the serum levels, the growth of cells is dependent on the supply of exogenous thymidine, and therefore, under these conditions, TK activity should play an important role in the pathway leading to dTTP formation for DNA synthesis and repair (39). Therefore, in cells such as hemopoietic BAF3 cells that are strictly dependent on growth factors for cell viability and proliferation, regulation of TK activity by IL-3 (11) could be an important event in the maintenance of balanced dNTP pools when the extracellular concentration of folic acid is comparable with that of serum.

Due to the complexity of dNTP metabolism and the multiple allosteric mechanisms involved in this metabolism, at present it is difficult to identify the critical variable that induces apoptosis during dNTP pool imbalances. The fact that inhibition of TK activity by IL-3 (11) could be an important event in the maintenance of balanced dNTP pools when the extracellular concentration of folic acid is comparable with that of serum.

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