Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 126-132

Bcl-2 ONCOGENE PROTECTS A BONE MARROW-DERIVED PRE-B CELL LINE FROM 5'-FLUOR,2'-DEOXYURIDINE-INDUCED APOPTOSIS+

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Received May 13, 1993

The *bcl-2* protooncogene has been shown to protect haemopoietic precursors from programmed cell death after the removal of interleukin-3 (IL3). In the present report we show evidence that overexpression of *bcl-2* in the pre-B-cell line BAF3 protects cells from apoptosis induced by treatment with the thymydilate synthase inhibitor 5'-fluor,2'-deoxyuridine (FDUR) in the presence of IL-3. Dose-response experiments analyzing the dependence of cell death on drug concentration indicated a marked resistence of BAF3^{bcl-2} to FDUR treatment. Cleavage of DNA into oligonucleosome-length fragments, a characteristic of apoptosis, was observed in BAF3 cells and inhibited in the cells overexpressing *bcl-2*. We have determined variations in the dATP and dTTP pools after FDUR treatment. Interestingly, no differences were found between both cells in the kinetics of changes in dNTP pools. Therefore, the protective effect of the *Bcl-2* protein on apoptosis induced by dNTP unbalance must be ascribed to a step downstream of perturbations in the synthesis of DNA precursors and before activation of endonucleolytic cleavage of chromatin.

Apoptosis is a form of cell death that has been observed in a number of physiological circumstances including the selection of B and T lymphocytes (1) and also in different cell systems after

+This work was supported in part by the Cancer Research Campaign and by a grant from CICYT (SAL91-0411).

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0006-291X/93 $4.00

cytokine deprivation (2). It involves the activation of an endonuclease that cleaves DNA into oligonucleosome-size fragments (3). Different antineoplastic agents acting on their target cells by inhibiting DNA precursors synthesis, have been reported to kill cells by a mechanism involving programmed cell death (4-6) and alterations in dNTP pools balance has been suggested to be one of the events leading to cell death in several cell models (4, 7, 8).

The *bcl-2* protooncogene product is a 26-kDa protein expressed in the inner mitochondrial membrane and the nuclear envelope (9,10). It was originally isolated from the translocation t(14;18) of a human follicular B cell lymphoma (11). Despite the fact that its molecular role still remains unknown, when overexpressed, *bcl-2* has been reported to prevent from programmed cell death in several cell and animal models (12). In certain cells its expression seems to be regulated by cytokines (13).

The above findings prompted us to analize the effect of dNTP unbalance -using the thymidilate synthase (TS) inhibitor and antineoplastic drug FDUR-, on the viability and DNA integrity of hemopoietic cells overexpressing the *bcl-2* oncogene. We have observed that such cells were more resistant to the induction of DNA fragmentation and cell death by FDUR than control BAF3 cells even in the continuous presence of IL-3. Since the primary biochemical effect of FDUR in the cell is well known, the results could provide insight into the molecular function of *bcl-2* in the protection from programmed cell death.

MATERIALS AND METHODS

Materials. RPMI 1640 and fetal bovine serum (FBS) were obtained from Gibco Europe. 5'-fluor,2-deoxyuridine was purchased from Sigma. Methotrexate was from Cyanamid Iberica, Division Lederle (Spain). Deoxy[8-^{3}H]adenosine 5'-triphosphate (24 Ci/mmol) and thymidine-5'-triphosphate [methyl-^{3}H] (53 Ci/mmol) were from Amersham (UK) and ICN Biomedicals, respectively.

Cell cultures. Murine IL-3-dependent BAF-3 cells (14) were maintained in culture in RPMI medium containing 10% fetal bovine serum, 1 mM glutamine and 5-10% conditioned medium (CM) from the IL-3-producing cell line Wehi-3B. Cell viability was determined by trypan blue dye exclusion .

Analysis of DNA fragmentation in agarose gels. $2x10^{6}$ cells were lysed with 10 mM Tris HCl pH 7.5, 1 mM EDTA, 0.2% Triton X-100

and fragmented DNA was prepared from the 13000xg supernatant after incubation overnight in a lysing buffer containing 10 mM Tris HCl pH 8.0, 40 mM EDTA, 120 mM ClNa, 0.8 % SDS, 0.16 mg/ml proteinase K. DNA was extracted once with phenol, once with chlorophorm:isoamylalcohol (49:1), the aqueous phase was made 150 mM with NaCl and precipitated with 2 vol of ethanol at -20°C overnight. Pellets were air-dried and resuspended in 50 μl of distilled water and incubated for 1 hour at 37 °C with 25 μg/ml of RNAse A.

Horizontal electrophoresis of DNA was performed in 1 % agarose gel with 90 mM Tris HCl, 90 mM boric acid, 2 mM EDTA, pH 8.0 as running buffer (TBE buffer). DNA was visualized after electrophoresis by ethidium bromide staining.

Deoxyribonucleoside triphosphate (dNTP) pool assay. Preparation of cell extract was essentially as described by Garret & Santi (15) with slight modifications. Briefly, 2-5 millions BAF3 cells were washed with 10 ml ice cold PBS. The pellet was resuspended in 50 μl PBS and cells were lised with 0.5 M $HClO_4$ for 30 minutes on ice. Precipitated material was discarded by centrifugation, the supernatant was neutralized with 4 M KOH/0.4 M KH_2PO_4, and kept at -20°C until use after discarding the undisolved salt. The deoxyribonucleotides were determined by a modification of the DNA polymerase assay (16). The concentrations of dNTP were stimated from calibration curves of known picomole amounts of pure standards.

Generation of bcl-2 expressing cells. A 1.9 Kb human *bcl-2* cDNA fragment was use for the generation BAF3 cells overexpressing *bcl-2*. Further details are given in ref.17.

RESULTS

Cultures of the bone-marrow derived pre-B cell line BAF3 depend on the presence of IL-3 for growth and survival and die rapidly by apoptosis when deprived of this factor (2). In these experiments we have determined the effect of the TS inhibitor FDUR on the viability of BAF3 cells maintained continously in the presence of IL3 and compared this with BAF3 cells overexpressing the Bcl-2 protein (17), also maintained in medium with IL-3. Control BAF3 cells were treated with 5 μM FDUR and samples were collected at different times to measure cell viability. Cell survival was clearly decreased after 15 hours of incubation with the drug and by 31 h only 25% of the population were viable cells (fig.1a) . No viable cells were detected after 55 hours. A distinct situation was observed with $BAF3^{bcl-2}$ cells treated with FDUR, where at 24 and 31 hours of treatment with the inhibitor cell survival was 94 and 88% respectively. At the latest time tested, 55 hours, no

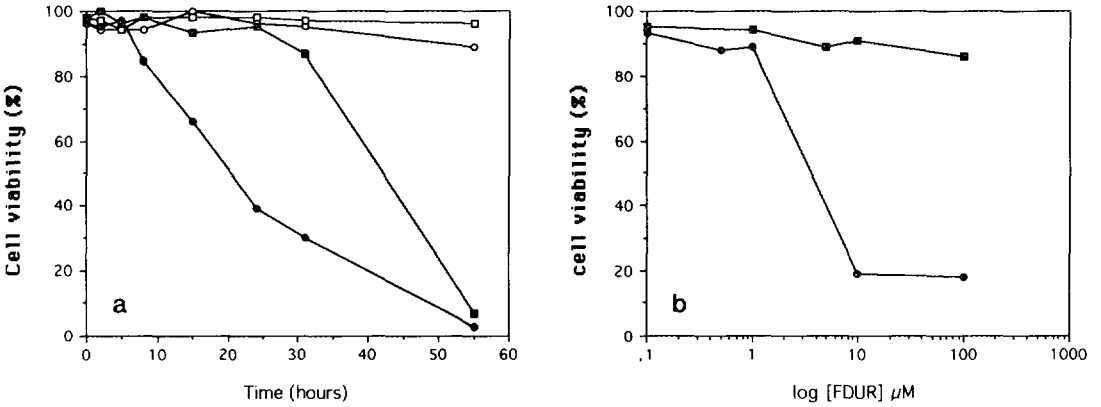

Figure1. Effect FDUR on the viability of BAF3 and BAF3^{bcl-2} cells. Cells cultured in RPMI with 10% foetal bovine serum and 10% conditioned medium from the IL-3 producer Wehi-3B cell line were counted at different times after treatment with 5 μM FDUR (a) or treated for 15 hours with various doses of FDUR (b). (○) BAF3, (●) BAF3+FDUR, (□) BAF3^{bcl-2}, (■) BAF3^{bcl-2} + FDUR.

difference in viability was observed between both types of cells.

Similar protection by *bcl-2* was observed in experiments where the cell viability of the cultures was assesed after treatment with various concentrations of inhibitor. In a representative experiment (Fig. 1b), BAF3^{bcl-2} cells were completely resistant to the action of up to 100 μM FDUR for 22 hours while BAF3 viability was reduced to 18%.

Since the most characteristic biochemical event during programmed cell death is the cleavage of chromatin into oligonucleosome-length fragments, we analyzed DNA fragmentation in BAF3 and BAF3^{bcl-2} cells treated with FDUR or methotrexate (a dihydrofolate analog that also inhibits dTTP syntesis). Results presented in figure 2 clearly show that treatment of BAF3 cells with both inhibitors induced DNA fragmentation in a way characteristic of apoptosis. However, in cells overexpressing Bcl-2, DNA fragmentation was markedly inhibited. In BAF3 cells, chromatin fragmentation could be observed as early as 5 hours after incubation with the drugs (not shown), time at which loss of viability was not observed. The effect of methotrexate on BAF3 and BAF3^{bcl-2} cell survival was very similar to that elicited by FDUR (results not shown).

The molecular mechanism of action of FDUR is known to be the inhibition of TS activity, that leads to an unbalance in dNTP

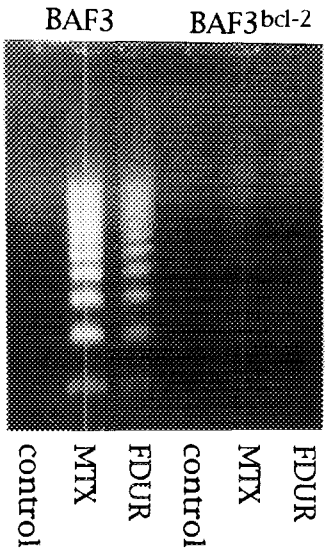

Figure 2. *Bcl-2* overexpression protects BAF3 cells from stimulation of DNA fragmentation. Analysis of DNA fragmentation was performed as described under Materials and Methods with cells treated for 15 hours with either 5μM FDUR or 4μM methotrexate.

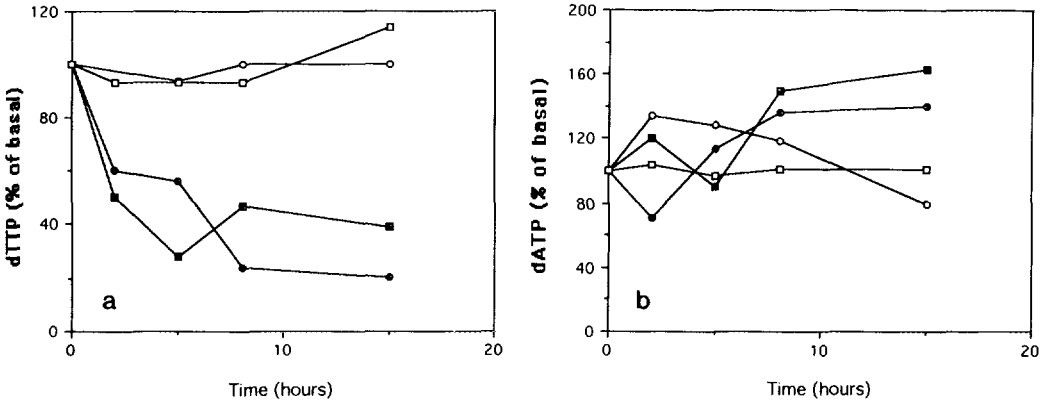

Figure 3. dATP and dTTP pools variations in BAF3 cells after FDUR treatment. The figure is from one representative experiment. dATP and dTTP levels were determined as described under Materials and Methods. Basal levels for dATP were 5.5±0.5 and 5.9±1.3 pmol/10^6 cells for BAF3 and BAF3^{bcl-2} cells, respectively. For dTTP the values were 20.0±1.5 and 15±2.2 pmol/10^6 cells in BAF3 and BAF3^{bcl-2} cells, respectively. These values are from at least three separate experiments. (○) BAF3, (●) BAF3 + FDUR, (□) BAF3^{bcl-2}, (■) BAF3^{bcl-2} + FDUR.

pools and eventually to the death of the cell by a yet unknown pathway. As *bcl-2* deregulation blocks programmed cell death induced by FDUR one possibility was that the *bcl-2* protein somehow prevented the dNTP unbalance elicited by the drug. This was not the case since no differences were found between BAF3 and BAF3^{bcl-2} cells in the kinetics of depletion of dTTP following the treatment with FDUR (fig. 3a), and no significant variations were detected in the dATP pool after treatment with the inhibitor (fig. 3b). Similar results were found in experiments using methotrexate to alter dNTP pools (not shown).

DISCUSSION

Previous observations have indicated that many chemotherapeutic drugs are capable of initiating pathways leading to apoptosis (4-6). Because deregulated *bcl-2* expression have been widely reported to interfere with programmed cell death (17-20) we decided to determine whether overexpression of the oncogene in the hemopoietic cell line BAF3 could render these cells more resistant to the action of the antineoplastic drugs FDUR and methotrexate. Our findings indicate that *bcl-2* can markedly delay inhibitor-induced apoptosis in BAF3 cells, including the chromatin breakage into oligonucleosome-length fragments.

Our previous data have shown that DNA damage induced by irradiation, etoposide or cisplatin caused the induction of apoptosis in BAF3 cells deprived of IL-3 (17). IL-3 or *Bcl-2* overexpression could inhibit apoptosis induced by these DNA damaging agents. In contrast apoptosis induced by FDUR or methotrexate occurs in the presence of IL-3 and can only be inhibited by *Bcl-2* overexpression. This probably means that DNA damaging agents and the TS inhibitor induce apoptosis by different mechanisms both of which can be inhibited by *Bcl-2* overexpression. These results could also suggest that the function of cytokines as survival factors in hemopoietic BAF3 cells is not likely to be mediated by endogenous bcl-2 regulation.

Perturbation of dNTP pool have been previously suggested to trigger DNA fragmentation and cell death in mouse leukemia cells (4), mouse mammary tumor cells FM3A (7) and mouse thymocytes (8). In order to define the pathway by which *Bcl-2* protein blocks programmed cell death we determined the alterations in dATP and dTTP pools in deregulated and control BAF3 cells and we did not find any difference in the levels of dNTP between both type of cells

that could account for their contrasting response to FDUR or methotrexate. 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, as suggested by others (4), perhaps by inhibiting a step close to the activation of the enzyme(s) that cleaves DNA. The lack of protection of cell viability by Bcl-2 after 55 h incubation with FDUR could be ascribed to a loss of effectiveness of the protein in preventing apoptosis in the long term as has been already described during negative selection of thymocytes (20).

dNTP pools balance has been shown to be essential for fidelity in DNA synthesis in dividing cells (21) and inhibition of DNA synthesis can be sufficient to cause cell death (6). Unbalance in deoxynucleotides may therefore serve as a signal to the cell to initiate a death program. This suggests that the cells commit themselves to the apoptotic pathway because an inaccurate DNA synthesis or repair (due to inadequate dNTP levels) could lead to mutations in the genome with harmful consequences (tumorogenesis) for the tissue or even for the whole organism.

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