Apoptosis Induction in Lymphoma Cells: Thiol Deprivation versus Thiol Excess

(apoptosis induction / thiol deprivation / thiol excess / lymphoma cells)

J. KOVÁŘ, H. ŠTÝBROVÁ, J. TRUKSA, K. SPĚVÁKOVÁ, T. VALENTA

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Abstract. We studied the effects of thiol availability on apoptosis induction in B-cell lymphoma 38C13, T-cell lymphoma EL4, and also other cells. Compounds with a free SH group are required for survival and growth of 38C13 cells but not of EL4 cells. Thiol deprivation (2-mercaptoethanol concentrations about 0.3 μM and lower) induced apoptosis in 38C13 cells. On the other hand, thiol excess (2-mercaptoethanol concentrations higher than 300 μM) induced apoptosis in 38C13 cells and EL4 cells as well as in other cells (e.g. Raji, HeLa). L-cystine and non-thiol antioxidant ascorbic acid were unable to support survival of 38C13 cells. Ascorbic acid induced cell death at concentrations higher than 600 μM. Thiol cross-linking compound diamide (100 μM and higher) abrogated the survival-supporting effect of 2-mercaptoethanol (50 μM). Apoptosis induction by thiol deprivation and by thiol excess was not directly related to a specific significant change in the p53 level or p53 activation. Apoptosis induction by thiol excess was associated with a certain decrease in the Bcl-2 level while the Bax level did not change. We conclude that both thiol deprivation and thiol excess can induce apoptosis in lymphoma cells. Apoptosis induction by thiol deprivation is specifically related to the presence of a free SH group. However, apoptosis induction by thiol excess does not seem to be specifically related to the presence of a free SH group. It probably results from the excess of a reductant. Apoptotic control protein p53 does not seem to play a significant role in apoptosis induction either by thiol deprivation or by thiol excess.

Apoptosis is a genetically encoded programme of cell self-destruction. It plays a significant role in many physiological processes such as those involved in development or those related to the function of the immune system. Apoptosis also occurs in tumour cells responding to therapy (Thompson, 1995; Jacobson et al., 1997; Raff, 1998). Thus, the factors involved in control of apoptosis are of particular interest.

Received October 19, 2001. Accepted January 28, 2002.

This work was supported by grant 301/97/1029 and by grant 301/01/0041 from the Grant Agency of the Czech Republic.

Corresponding author: Jan Kovář, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Tel.: +420 (2) 41062637; fax: +420 (2) 44471707; e-mail: kovar@biomed.cas.cz.

Abbreviation: PBS – phosphate-buffered saline.

Proteins of the Bcl-2 family such as antiapoptotic Bcl-2 and proapoptotic Bax or protein p53 seem to play an important role in control of apoptosis induction. On the other hand, changes in mitochondrial function, particularly those related to the change of mitochondrial membrane permeability, seem to be decisive for switching on the cellular apoptotic programme (Adams and Cory, 1998; Green and Reed, 1998; Gross et al, 1999). The redox status of the cell very likely plays an important role in these decisive events (Sato et al., 1995; Marchetti et al., 1997; Falk et al., 1998; Hall, 1999). Therefore, thiol antioxidants may play a significant role in apoptosis regulation and thus also in strategies concerning cancer treatment.

It was shown earlier that 2-mercaptoethanol was required for growth of B cells (Ishii et al., 1981). More recently, it has been demonstrated that 2-mercaptoethanol and other thiol compounds promote survival and prevent apoptosis in various lymphoid cells (Sato et al., 1995; Delneste et al., 1996; Kinoshita, 1997; Falk et al., 1998; Neumann, 1998), and also in other types of cells (Castro-Obregon and Covarrubias, 1996; Aoshiba et al., 1999, Jayasurya et all., 2000, Yang et al., 2000). It has also been demonstrated that thiols can inhibit apoptosis induced by other apoptotic stimuli such as glucocorticoids and irradiation (McLaughlin et al., 1996; Marchetti et al., 1997; Mirkovic et al., 1997) or viruses (Lin et al., 1995). Antiapoptotic thiol effects are supposed to be based on the presence of a free SH group (Delneste et al., 1996; Deas et al., 1997; Falk et al., 1998).

It has been found that some thiol compounds can inhibit proliferation of human leukaemic cells (Jeitner et al., 1998). It has also been found that exposure to some thiols at relevant concentrations can even kill cells of non-haematopoietic origin (Takagi et al., 1974; Held and Melder, 1987; Held and Biaglow, 1994). The authors suppose that thiol cytotoxicity results from the generation of H_2O_2. However, the cell killing was not related to apoptosis in these papers.

Mouse B-cell lymphoma 38C13 was found previously to be highly sensitive to apoptosis induction by iron deprivation (Kovar et al., 1997). Later, the lymphoma was also found to be highly sensitive to 2-mercaptoethanol withdrawal from culture media while mouse T-cell lymphoma EL4 was found to be completely

resistant. Therefore, we decided to study the effects of thiol availability on apoptosis induction specifically in lymphoma cells, employing as a model lymphoma 38C13 versus lymphoma EL4.

Material and Methods

Chemicals and reagents

2-mercaptoethanol was from Fluka (Buchs, Switzerland), dithiothreitol was from Bio-Rad (Hercules, CA), L-cysteine, L-cystine, ascorbic acid and diamide were from Sigma (St. Louis, MO). Mouse monoclonal antibody PAb 240 against mouse p53 from Calbiochem (La Jolla, CA), mouse monoclonal antibody HZ 52 against mouse $p21^{CIP1/WAF1}$ from Neo Markers (Fremont, CA), Syrian hamster monoclonal antibody 3F11 against mouse Bcl-2 from Pharmingen (San Diego, CA) and rabbit polyclonal antibody (Sc-562) against mouse Bax from Santa Cruz (Santa Cruz, CA) were used.

Cells and culture conditions

The mouse B-cell lymphoma 38C13 and the mouse T-cell lymphoma EL4 were obtained from Prof. J. Kemp (University of Iowa, Iowa City, IA). For some confirmatory experiments, human Raji and HeLa cells were employed. The human Burkitt lymphoma Raji was obtained from Prof. G. Klein (Karolinska Institutet, Stockholm, Sweden), and human cervix carcinoma HeLa was obtained from Dr. A. Cvekl (Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic). The cell lines were routinely tested for mycoplasma contamination using the fluorescent Hoechst 33258 staining method (Chen, 1977).

Defined serum-free culture media were used. The basic medium was RPMI 1640 containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), Hepes (15 mM), iron-saturated human transferrin (5 μg/ml), ethanolamine (20 μM), ascorbic acid (20 μM), hydrocortisone (5 nM) and 11 trace elements, as described previously (Kovář, 1988; Kovář and Franěk, 1989). Cells were routinely maintained in the basic medium supplemented with 2-mercaptoethanol (50 μM). In experiments, the effects of the basic medium supplemented with various concentrations of 2-mercaptoethanol, L-cysteine, L-cystine, dithiothreitol, ascorbic acid and diamide were tested. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Cell growth and survival analysis

Cells maintained in the basic medium supplemented with 50 μM 2-mercaptoethanol were harvested by low-speed centrifugation, washed with the basic medium and then seeded at 40 x 10^3 (20 x 10^3) cells/100 μl of medium into wells of a 96-well plastic plate. Cell growth and survival in the basic medium and in the basic medium with tested concentrations of 2-mercaptoethanol, L-cysteine, L-cystine, dithiothreitol, ascorbic acid and diamide were assessed after 24 h of incubation. The number of living cells was determined by haemacytometer counting after staining with trypan blue.

Propidium iodide staining analysis

Cells previously grown in the basic medium supplemented with 50 μM 2-mercaptoethanol were harvested by low-speed centrifugation, washed with the basic medium and seeded at 400 x 10^3 cells/ml of medium into plastic culture flasks. The effect of control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (basic medium without 2-mercaptoethanol) and thiol excess (basic medium with 1000 μM 2-mercaptoethanol) was tested. After 0, 8, 16 and 24 h of incubation, the cells were harvested by low-speed centrifugation and stained. Briefly, approximately 4 x 10^6 cells per sample were washed twice with 2 ml of phosphate-buffered saline (PBS) and then fixed in 2 ml of 70% ethanol for 1 h at 4°C. Fixed cells were centrifuged and washed with 2 ml of PBS. The cell pellet was treated with 1 ml of 0.1% Triton X-100 at 4°C for 3 min and centrifuged. Supernatant was removed and 1 ml of RNAse (100 U/ml of PBS) was added. After a 10-min incubation at room temperature, the samples were spun and supernatant was aspirated. Propidium iodide (1 ml, 50 μg/ml) was added and the samples were incubated in the dark at 4°C for 1 h. Stained cells were analysed in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

DNA fragmentation analysis

Cells grown in the basic medium supplemented with 50 μM 2-mercaptoethanol were harvested by low-speed centrifugation, washed with the basic medium and seeded at 400 x 10^3 (800 x 10^3) cells/ml of medium into plastic culture flasks. The effect of control conditions (basic medium with 50 μM 2-mercaptoethanol), thiol deprivation (basic medium without 2-mercaptoethanol) and thiol excess (basic medium with 1000 μM 2-mercaptoethanol), as well as the effect of basic medium with employed concentrations of L-cysteine, L-cystine and dithiothreitol, was tested. After 16 h (22 h) of incubation, the cells were harvested by low-speed centrifugation and analysed. Briefly, 10^6 cells per sample were lysed in 400 ml of lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100) for 5 min at 4°C. Lysates were centrifuged at 13000 x *g* for 15 min at 4°C. DNA in the supernatant was precipitated overnight at -20°C by addition of 700 μl of 96% ethanol and 120 μl of 5 M NaCl. Samples were centrifuged again at 13000 x *g* for 15 min at 4°C. The pellet was washed twice with 200 ml of 70% ethanol and dried at room temperature. The dried pellet was dissolved in 20 μl of TE buffer (10 mM Tris, 1 mM EDTA) at 65°C. After cooling to room temperature, 2 μl of RNAse (100 U/ml of PBS) and 2 μl of proteinase K (2 mg/ml) were added and samples were incubated for 10 min at room temperature. Finally, 6 μl of loading dye (40 mM Tris, 20 mM sodium acetate,