



Short Communication

Changes in Glucocorticoid-Induced Apoptosis and in Expression of Bcl-2 Protein During Long-Term Culture of Thymic Lymphoma

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Abstract. Lymphomagenesis is a multistep process progressively freeing transformed thymocytes from external regulatory signals, i.e. thymic developmental program controlling growth, differentiation or apoptosis. Here we report that cells of thymic lymphoma overexpressing Ras/Raf proteins, initially resistant to TCR-dependent apoptosis but sensitive to dexamethasone- and etoposide-induced cell death, became insensitive to dexamethasone after long-time culture. That transition correlated with a strong increase in the expression of the anti-apoptotic Bcl-2 protein. Interestingly, lymphoma cells were still sensitive to p53-mediated apoptosis induced by etoposide. It suggests that the anti-apoptotic activity of Bcl-2 is correlated with a resistance to glucocorticoid-induced apoptosis but not to p53-mediated apoptosis. The sequence of mutations in the process of lymphomagenesis seems to be composed of at least 3 main hits which equip the cells with independence from external mitogenic signals (activation of Ras/Raf), resistance to inducers of apoptosis (activation of Bcl-2) and generation of cellular heterogeneity (deletion of p53) important in tumor progression.

Key words: thymic lymphoma; Bcl-2; dexamethasone; apoptosis; long-term culture.

Introduction

Signals from T cell receptors (TCR) direct the processes of positive and negative selection resulting in the T cells' maturation or apoptosis of thymocytes. Their anterior pre-TCR forms⁵ govern the process of maturation from the CD4⁺8⁺ to the CD4⁺8⁺ stage of differentiation accompanied by the replicative cycles that yield a 100-fold increase in thymic cellularity¹. Various agents induce apoptosis of thymocytes via independent signaling pathways^{3, 9}.

The TCR-dependent calcium-mediated signaling

pathway operates antigen-induced deletion of potentially autoreactive T cells². Antigenic stimulation or calcium ionophore delivers signals through the TCR-dependent pathway.

Immature thymocytes are sensitive to apoptosis induced by glucocorticoids (dexamethasone). Signals of positive selection trigger an increase in the expression of the anti-apoptotic Bcl-2 protein and protect cells against apoptosis induced by dexamethasone⁴.

Other pathways of apoptosis are triggered via the p53 protein as a response to damaged DNA⁶ by geno-

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toxic factors such as γ -irradiation or etoposide (topoisomerase inhibitor).

In a previous study we found that cells of thymic lymphomas from TCR anti-HY transgenic mice were resistant to TCR-dependent calcium-mediated induction of apoptosis but were sensitive to dexamethasone and etoposide¹⁰.

The initial stages of the neoplastic transformation of immature thymocytes could be defined as an accumulation of lesions leading, among other things, to a break in the TCR-dependent pathway of antigen-induced apoptosis. This could protect thymocytes with potentially autoreactive TCRs as a target for the next stages of lymphomagenesis. In parallel, overexpression of proteins from the Ras/Raf/MEK/ERK pathway governing proliferation would secure propagation of transformed cells. In fact, we have shown overexpression of Ras and Raf in thymic lymphomas⁷.

Here we report results showing that lymphoma cells during long-term culture accumulate additional abnormalities leading to overexpression of the anti-apoptotic Bcl-2 protein and resistance to dexamethasone-induced apoptosis.

Materials and Methods

Thymocytes. Thymocytes were isolated from C57BL/6/Boyliw mice obtained from the colony at the Institute of Immunology and Experimental Therapy, Wrocław. Suspensions of thymocytes were prepared in RPMI medium by pressing the thymus through a fine nylon mesh.

Lymphoma cells. VIII clone (derived from line VIII Lv) of thymic lymphoma of mice with transgenic TCR anti-HY¹⁵ were cultured (37°C, 5% CO₂) in 24-well Costar tissue culture plates in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20 μ M β -mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% FCS. Jurkat E6.1, human lymphoma, and EL4, mouse lymphoma, T cell lines (Culture Collection of the Institute of Immunology and Experimental Therapy, Wrocław, Poland) were maintained in IMDM or RPMI with 10% FCS, respectively.

Cells of the VIII clone were taken up to analyses after 3 or 6 month of cultivation and were denoted as VIII/k (after 3 months) or VIII/d (after 6 months).

Analysis of apoptosis. Thymocytes and lymphoma cells were cultured for 20 h (37°C, 5% CO₂) in 96-well Costar flat bottom tissue culture plates in IMDM with 10% FCS (2×10^5 cells per well) and with a continuous presence of one of the applied inducers of apoptosis

(Sigma): 1 μ M of dexamethasone, 0.5 μ M of A23187, 1 μ g/ml of ionomycin or 50 μ M of etoposide. Cells treated with the solvent only (0.05% DMSO in IMDM with 10% FCS) were taken as controls in all experiments. After treatment, the cells were washed in phosphate buffered saline (PBS), fixed with 70% ethanol for an hour at 4°C and DNA content was evaluated by the method using propidium iodide and flow cytometry¹² as described in our previous paper¹⁰. Apoptosis was quantified as percentages of cells with hypodiploidal DNA content.

Western blot analysis of Bcl-2 family expression. Single-cell suspensions were lysed in a lysis buffer containing 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 15 μ g/ml aprotinin and 15 μ g/ml leupeptin for 30 min on ice, and centrifuged for 10 min at 13 000 RPM to remove nuclei¹³. The protein concentration in supernatants were determined by a BCA protein assay with bicinchoninic acid¹⁴. The supernatants were mixed with Laemmli's sample buffer without 2-mercaptoethanol and boiled for 8 min. Aliquots containing 50 μ g of protein were loaded in each lane of 12% SDS-polyacrylamide gel and transferred to nitrocellulose filters using a Mini Protean Cell II (Biorad). The membranes were blocked in 10% non-fat milk in PBS-T (containing 0.1% Tween 20) for 1 h at room temperature or overnight in PBS with 10% non-fat milk at 4°C. Preblocked blots were probed with rabbit anti-Bcl-2 antibody (Santa Cruz) in PBS-T (1% skim milk) for 1 h at room temperature followed by washes in PBS-T (4 times for 10 min in PBS-T). Donkey anti-rabbit immunoglobulin-horseradish peroxidase linked whole antibody (Amersham) was used as secondary antibody and reacted with the blots for 1 h at 1: 5000 dilution at room temperature. The blots were rinsed 4 times for 10 min in PBS-T and developed using the ECL Western blot detection system (Amersham). Prestained molecular weight markers were included in every experiment (Sigma).

Results and Discussion

Cells of clone VIII, analyzed here, represent lymphoma originating from immature thymocytes. Phenotypic and functional analysis of the lymphoma cells indicated that they originated from the stage of pre-TCR-dependent transition of CD4⁻8⁻ to CD4⁺8⁺ thymocytes¹⁵.

Cells were maintained in long-term cultures and tested for changes in susceptibility to inducers of apop-

Table 1. Sensitivity of VIII thymic lymphoma cells after 3 months (VIII/k) and 6 months (VIII/d) of cultivation to dexamethasone-, calcium-, or etoposide-mediated induction of apoptosis

	Cells with hypodiploidal DNA [%]		
	thymocytes	VIII/k	VIII/d
Control (0.05% DMSO)	15 ± 13*	4 ± 4	4 ± 5
Dexamethasone, 1 µM	83 ± 5	64 ± 13	8 ± 8
A23187, 0.5 µM	66 ± 15	22 ± 18	28 ± 30
Etoposide, 50 µM	80	91	95

* Mean ± SD from 4 experiments.

tosis and expression of Bcl-2 protein. VIII/k and VIII/d were both resistant to calcium ionophore A23187 or ionomycin (not shown) and sensitive to etoposide, but responded differentially to treatment with dexamethasone (Table 1). VIII/k (3 months in culture) were sensitive to dexamethasone, while VIII/d (6 months in culture) were resistant to dexamethasone in 4 independent experiments. Lysates prepared from VIII/k and VIII/d cells were analyzed for changes in expression of Bcl-2. We found a lack of expression of Bcl-2 protein in dexamethasone-sensitive VIII/k cells and its strong expression in VIII/d cells (Fig. 1), resistant to dexamethasone-induced cell death but sensitive to etoposide treatment. This suggests that the anti-apoptotic activity of Bcl-2 is correlated with resistance to glucocorticoid-induced apoptosis but not to p53-mediated apoptosis.

**Fig. 1.** Western blot analysis of the Bcl-2 protein expression. Shown are results for Jurkat (Jur), EL4, thymocytes (Thc) and tested thymic lymphoma cells VIII after 3 months (VIII/k) and 6 months (VIII/d) of cultivation

Lymphomagenesis is a multistep process progressively accumulating genetic mutations and, in consequence, leads to the release of the cell cycle of transformed thymocytes from TCR-dependent regulatory signals and developmental program controlling growth, differentiation or apoptosis.

During the progression of leukemogenesis, lymphoma cells become resistant to dexamethasone, which induces glucocorticoid-dependent cell death. It has been shown here and by others that a rise in the expression of Bcl-2 protein in lymphoma cells increases resistance of the cells to dexamethasone-induced apoptosis¹¹. It should be noted that the cells were still sensitive to etoposide-induced apoptosis.

In the further stages of neoplastic progression, possible mutations or deletion of p53 genes could grant

lymphoma cells with a resistance to cell death induced by etoposide and other genotoxic agents and would increase their ability for generation of cellular heterogeneity⁸ in expanding lymphoma.

It seems that the process of lymphomagenesis could be drawn as a sequence of at least 3 key events equipping the cells with: independence from external mitogenic signals (Ras/Raf overexpression), resistance to inducers of apoptosis (Bcl-2 overexpression) and in the last step generation of cellular heterogeneity (p53 deletion), important in tumor progression. Hence, VIII/k and VIII/d cells can be regarded as a model for the study of the first and second stages of lymphomagenesis.

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