Dexamethasone induces apoptosis in human T cell clones expressing low levels of Bcl-2

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Abstract

Previous results of ours have demonstrated that the same clonotype can express both a sensitive and a resistant phenotype to Dex-mediated PCD induction depending on its cell cycle phase. In particular, we demonstrated that human T lymphocytes, arrested in the G0/G1 phase of the cell cycle, are susceptible, while proliferating T cells are resistant to Dex-mediated apoptosis. In this paper, we have further characterized the sensitive and resistant phenotypes and investigated whether a different expression of the apoptotic genes Fas, FasL, Bcl-2, Bcl-x and Bax is involved in the regulation of Dex-mediated apoptosis. The results show that the amount of Bcl-2 expression, that changes during cell cycle phases, determines susceptibility or resistance to apoptosis induced by Dex. In fact, undetectable expression of Bcl-2 in sensitive cells favors Dex-mediated apoptosis while high expression of Bcl-2 in proliferating cells counterbalances apoptosis induction. Moreover, the addition of exogenous IL-2, in the presence of Dex, fails to up-regulate Bcl-2 expression and to revert Dex-mediated apoptotic phenomena.

Keywords: human T lymphocytes; Dex; IL-2; Bcl-2; apoptosis

Abbreviations: Dex, dexamethasone; PI, propidium iodide; ASP, apoptosis sensitive phenotype; ARP, apoptosis resistant phenotype

Introduction

Apoptosis plays an important role in shaping and maintaining the T cell repertoire. In the thymus, this phenomenon is fundamental for the deletion of autoreactive T cells and the maintenance of T cell tolerance.¹ T cells that escape deletion in the thymus can be tolerated in the periphery, and apoptosis of peripheral T cells represents an important mechanism in ensuring the maintenance of immunological memory and to

avoid an excess of immune response.² Glucocorticoids are included among the factors known to induce apoptosis and regulate both central and peripheral tolerance in T cells.^{3–5} However, although it is well known that the complex glucocorticoid-glucocorticoid receptor (GR) results in the expression or repression of genes with a glucocorticoid responsive element (GRE), that in turn may regulate other genes involved in T cell activation, less is known about its effect on genes that are required for apoptosis induction.^{6–8}

Fas and Bcl-2 proteins identify two families of genes directly implicated in lymphoid T cell apoptosis.^{9–12} Fas is a transmembrane receptor belonging to the tumor necrosis factor/nerve growth factor (TNF/NGF)-receptor superfamily¹³ that induces apoptosis when triggered by agonistic antibodies¹⁴ or by its ligand, FasL.^{15,16} On the other hand, Bcl-2 is an intracellular membrane-associated protein family, whose members are differentially involved in increasing or decreasing susceptibility to T cell death.^{17,18} The members of the family, Bcl-2, Bax and Bcl-x, share two highly conserved regions called BH1 and BH2 domains through which they form active dimers.¹⁹ While Bcl-2 increases the survival of T cells in terms of growth factors deprivation and counteracts different kinds of apoptosis,¹⁷ Bax promotes apoptosis induction.²⁰ Hence the ratio of Bcl-2 to Bax, determining the amount of Bcl-2/Bax heterodimers *versus* Bax/Bax homodimers, influences susceptibility to apoptosis. The Bcl-x gene product exists in two forms: a long one, Bcl-x_L, and a short one, Bcl-x_S.²¹ Bcl-x_L inhibits apoptosis in many systems, while Bcl-x_S counters the protective effect of Bcl-2 or Bcl-x_L.²² Fas/FasL and Bcl-2 apoptotic gene families have been related to two mechanistically different forms of apoptosis that may occur in CD4⁺ T cells: Activation Induced Cell Death (AICD) and Programmed Cell Death (PCD).²³ In fact it has been shown that AICD can be avoided by blocking the interaction of Fas with FasL, while PCD can be stopped by adding exogenous interleukin-2 (IL-2) or by allowing expression of the survival genes Bcl-2 or Bcl-x_L.²⁴

Even if the intracellular pathways of PCD induction are under investigation and many pieces of the puzzle are still missing, it seems that the IL-2-rescue from apoptosis is due to an increase of the Bcl-2 protein. In fact following the binding of IL-2 with the IL-2 receptor (IL-2R), three distinct pathways are activated and co-operate to allow T cell survival and progression in the cell cycle.²⁵ The three pathways, mediated by a protein kinase cascade, activate Bcl-2, *c-myc* and *c-fos/c-jun* respectively and very recently a paper on IL-2 signaling has indicated the Akt kinase as the kinase responsible for Bcl-2 expression.²⁶ However, when PCD is triggered by glucocorticoids, the role of IL-2 in the inhibition of apoptosis and in the regulation of Bcl-2 expression has not been well established. In fact even if the IL-2 dependent cell line CTLL-2 is rescued from glucocorticoid-mediated apoptosis by IL-2,^{27,28} the effect

of IL-2 on normal lymphocytes, programmed to cell death, is less clear.

To elucidate whether a different expression of Bcl-2 can protect T cells from apoptosis mediated by Dexamethasone (Dex), a synthetic glucocorticoid, and whether IL-2 can influence this expression, we investigated Bcl-2, Bcl-x, Bax, Fas and FasL expression in a well characterized apoptosis-induction T cell system in our laboratory. We

analyzed the induction of Dex-mediated apoptosis in human mature T cells in a previous study and showed that the same cloneotype can express both a sensitive and a resistant phenotype to PCD induction depending on its cell cycle phase.²⁹ In particular we demonstrated that T lymphocytes, arrested in the G0/G1 phase of the cell cycle, are susceptible, while proliferating T cells are resistant to Dex-mediated apoptosis. In this paper, we

Figure 1 Identification of Apoptosis Sensitive Phenotype (ASP) and Apoptosis Resistant Phenotype (ARP). Four T cell clones (G12, G3, HC3 and HC6) arrested in G0/G1 (first line from left) or moving to S (third lane from left) phase of cell cycle were harvested and analyzed for their susceptibility to Dex-induced apoptosis. The results are expressed as cytofluorimetric analysis of DNA content

show that the level of Bcl-2 expression regulates the susceptibility or resistance to Dex-mediated PCD in resting or proliferating cells, respectively. Moreover, we have clearly pointed out the effect of exogenous IL-2 in regulating Dex-mediated PCD. In fact our results show for the first time that IL-2 fails to up-regulate Bcl-2 in Dex-treated resting T cells.

Results

Characterization of phenotypes sensitive or resistant to Dex-induced apoptosis in T cell cultures of the same clonotype

We previously demonstrated that Dex susceptibility of T cells to apoptosis is cell-cycle-dependent.²⁹ In this paper, to further investigate the differential susceptibility to Dex-induced apoptosis of resting *versus* activated T cells, we have defined the cellular parameters measured as DNA content that characterizes the sensitive or resistant phenotypes in a same clonotype. To this end, T cells clones were antigen

activated and cultured for 10–11 days in the presence of exogenous IL-2. Every 3–4 days, the cells were harvested and flow cytometric analysis performed. The data shown in Figure 1 clearly demonstrated that synchronous cell populations accumulated in the G0/G1 compartment, as those harvested after 10–11 days from antigen stimulation, were susceptible to Dex-induced apoptosis. In contrast, asynchronous growing cell populations with at least 15% of hyperdiploid cells, as those harvested after 4–7 days from antigen stimulation, were resistant. In fact, after 24 h of culture with Dex, the proportion of apoptotic cells was usually between 60–70% in the former population and less than 10% in the latter. Consequently, synchronous and asynchronous population defined the apoptosis sensitive phenotype (ASP) and apoptosis resistant phenotype (ARP), respectively.

IL-2 protects T cells from induction of PCD by growth factor withdrawal, but not by Dex

To determine whether IL-2 was able to protect T cells from Dex-mediated apoptosis, ASP was treated with Dex 10^{-7} M

Figure 2 IL-2 does not protect T cells from Dex-mediated apoptosis. ASP was treated (A and B) or not (C and D) with 10^{-7} M Dex for 24 h in the absence (A and C) or presence (B and D) of 20 U/ml IL-2. FACS analysis of apoptotic cells is reported as percentage in the evidenced regions

in the presence or absence of 20 U/ml of IL-2 for 24 h. As shown in Figure 2 FACS analysis reveals that both in the absence (panel A) and in the presence (panel B) of IL-2, Dex induces a massive cell death. However, the evidence that the addition of IL-2 to Dex-treated culture resulted in the reduction, although not significant, of apoptosis, led to the question of whether the protective effect of IL-2 was a direct effect on T cells cultured for 24 h in the absence of growth factors or an indirect effect on Dex-mediated apoptotic pathways. To answer this question we added IL-2 to Dex-untreated cultures and looked at the results. As shown in Figure 2 (panels C and D), the addition of IL-2 significantly reduced the spontaneous apoptosis which occurred in T cells cultured in the absence of IL-2. We can thus conclude that IL-2 rescues T cells from T cell growth factor deprivation and not from Dex-mediated apoptosis.

Dex sensitive cells have undetectable levels of Bcl-2

To understand why IL-2 failed to rescue ASP from Dex-mediated apoptosis we decided to perform a detailed examination of gene expression through RT-PCR analysis of some of the major genes involved in apoptosis. To evaluate gene expression before the formation of apoptotic bodies, mRNA was extracted after 8 h of incubation. The results of the assay, shown in Figure 3, clearly demonstrate that ASP have undetectable levels of Bcl-2 expression (panel A) and that neither Dex (panel B) nor Dex plus IL-2 (panel C) were able to modify it. Moreover, this analysis evidenced the lack of FasL and the presence of Bcl-x, Bax and Fas expression in the ASP, and the observed expression remained unmodified both in the presence of Dex and Dex plus IL-2.

Figure 3 ASP expresses undetectable levels of Bcl-2. ASP was cultured in the absence (A), in the presence of Dex (B) and of Dex+IL-2 20 U/ml (C). At the end of the incubation period mRNA was extracted and amplified for Bcl-2, Bcl-xL, Bax, Fas and FasL expression by RT-PCR. For normalization, β -actin was amplified in the same reaction tubes (548 bp)

Dex resistant cells have high levels of Bcl-2

To further investigate the causes of the differential sensitivity to Dex-mediated apoptosis, we performed RT-PCR analysis on ARP too. The analysis evidenced that the ARP population expressed high levels of Bcl-2 expression that were not modified by the presence of Dex in culture. The inability of Dex to induce apoptosis and the unmodified expression of Bcl-2 strongly suggested that Bcl-2 expression can regulate the resistance to Dex-mediated apoptosis. In Figure 4, the analysis of Bcl-x, Bax, Fas and FasL expression is also shown. It is interesting to note that all the analyzed genes were expressed in both Dex-treated or untreated cultures, with the exception of FasL. In fact, although ARP presented high levels of FasL, Dex strongly reduced this expression and this occurred regardless of the presence of IL-2.

IL-2 differentially regulates Bcl-2 expression depending on the presence of Dex

The above reported results on the ability of IL-2 to rescue ASP from IL-2 withdrawal, and not from Dex-mediated cell death, gave rise to the question 'does exogenous IL-2 exert its protective role by up-regulating Bcl-2'? The answer to this question can be useful not only to verify the effect of IL-2 on Bcl-2 expression in our model, but also to confirm the relationship between Bcl-2 level and susceptibility to Dex-mediated apoptosis. As Figure 5 shows, both immunofluorescence assay (panels A and B) and RT-PCR analysis (panel C) clearly demonstrated that IL-2 is able to up-regulate Bcl-2 expression and to favor the progression of T cells in the S/G2 cell cycle phase.

Figure 4 ARP expresses high levels of Bcl-2. ARP was cultured in the absence (A), in the presence of Dex (B) and of Dex+IL-2 20 U/ml (C). At the end of the incubation period mRNA was extracted and amplified for Bcl-2, Bcl-xL, Bax, Fas and FasL expression by RT-PCR. For normalization, β -actin was amplified in the same reaction tubes (548 bp)

Finally, to confirm that the failure of the protective role of IL-2 in Dex-mediated PCD, in cells accumulated in G0/G1 cell cycle phase and expressing undetectable Bcl-2 levels, was a general phenomenon and not restricted to the G12 T cell clone, we performed similar experiments using other CD4⁺ T clones. As can be seen in Table 1, IL-2 in the presence of Dex was not able to protect T cells from apoptosis induction in all the clones investigated.

Discussion

Starting from the observation that positive and negative regulators of apoptosis are the Bcl-2 and Fas gene families respectively,⁹⁻¹¹ we studied the expression of these genes and proteins in human T cell clones during the cell cycle and after Dex treatment. The main results may be summarized as follows: (i) both Bcl-2 and FasL upregulation is a consequence of T cell activation that persists during progression through the cell cycle phases, to decrease when the cells arrest in the G1 phase; (ii) Dex-mediated apoptosis occurs only in the G1

phase when Bcl-2 is down-expressed; and (iii) IL-2 fails to modify Bcl-2 expression and susceptibility to Dex-mediated apoptosis.

The Bcl-2 capacity to prolong cell survival has been well documented in many different systems.³⁰ In transgenic

Table 1 IL-2 is not able to rescue T cell clones from Dex-mediated PCD

	% Hypodiploid cells ^b	
	IL-2	Dex+IL-2
T cell clones ^a		
HC3	7%	72%
HC6	5%	66%
G3	9%	71%

^aThe HA-specific T cell clones HC3 and HC6 and the DR1-alloreactive T cell clone G3 were treated ON with or without Dex 10^{-7} M in the presence of 20 U/ml of IL-2. ^bApoptosis was analyzed by flow cytometry after PI staining. The values indicate the percentage of cells with a higher Side Scatter and containing nuclei with a lower DNA content

Figure 5 Bcl-2 is upregulated by IL-2. ASP was treated (B) or not (A) for 24 with IL-2 and both Bcl-2 protein expression and DNA content measured. In parallel RT-PCR analysis of untreated (ASP) or IL-2-treated (+IL-2) cells was performed. For DNA and Bcl-2 analysis, cells were permeated with methanol and double stained with anti-Bcl-2 antiserum (Y axis) and PI (X axis). For normalization in the RT-PCR analysis, β -actin was amplified in the same reaction tubes (548 bp)

animals, it has been observed that an increased expression of Bcl-2 protects cells from apoptosis induced by various factors such as glucocorticoids, radiation, stimulation by anti-CD3 monoclonal antibodies and calcium ionophores.^{31–33} The inverse relationship between Bcl-2 expression and susceptibility to glucocorticoids has also been demonstrated in normal and in transfected B cell lines.^{34–36} More recently, it has been reported that the transient overexpression of Bcl-2 in mouse and human T cell blasts did not block Fas-mediated apoptosis, whereas it inhibited glucocorticoid-mediated apoptosis.³⁷ Our results, showing that Bcl-2 protects T cells from Dex-mediated PCD, are in agreement with all the above-reported observations and stress that Bcl-2 expression, varying with the progression of the cell cycle, allows sensitive and resistant phenotypes to Dex-mediated apoptosis to be established.

Even if it has been demonstrated that IL-2 induces Bcl-2 expression, the ability of IL-2 to protect T cells from Dex-mediated apoptosis is still controversial. In fact, results obtained with the IL-2 dependent cell line CTLL-2 evidenced that the presence of IL-2 was inversely related to the level of apoptosis and directly related to the level of Bcl-2,^{28,38} but data with antigen-specific T cell lines are less clear.

In murine and rat models, it has been shown that IL-2 is able to rescue T cells from Dex-mediated apoptosis.^{39,40} However, since these are different from the human models, it is easy to imagine that they are also differentially regulated. In fact, both murine and rat cells are always susceptible to Dex-mediated PCD, while human T cells may present a susceptible or a resistant phenotype.²⁹

Our results show that once human T cells are arrested in the G0/G1 phase of the cell cycle and have down-regulated Bcl-2, they are susceptible to Dex induced PCD and addition of IL-2 is not able to rescue them from death.

IL-2 signals are transduced, in the T cell, through the activation of a cascade of protein phosphorylation.⁴¹ Recently, the work on the 'transduction of IL-2 anti-apoptotic and proliferative signals' by Ahmed and colleagues shows that the expression of Bcl-2 and *c-myc* depends on the activation of the PI 3-kinase/Akt pathway originating in the S region of the IL-2R β .²⁶ In fact, the authors demonstrate that the serine-threonine protein kinase encoded by the Akt proto-oncogene is activated by IL-2 and that the expression of a catalytically active Akt mutant promotes the expression of Bcl-2 and *c-myc* in cells that express an IL-2R β deleted of the S region. The inhibition of the IL-2 dependent tyrosine phosphorylation of several intracellular proteins by glucocorticoids in human T cells has been demonstrated by Paliogianni and colleagues.⁴² More recently, the same authors have reported that Dex inhibits the activation of the Ca²⁺/calmodulin-dependent protein kinase II (CaM kinase II) in primary human T cells stimulated with a combination of ionomycin and phorbol ester.⁴³ In particular, this study evidences that the inhibition of CaM kinase II autophosphorylation by Dex is due to the activation of protein phosphatase activity, suggesting that the inhibition of phosphorylation is the consequence of protein phosphatase induction more than

protein kinase inhibition. In this scenario, we can imagine that the binding of IL-2 to IL-2R activates a cascade of kinases that induces the survival gene Bcl-2, but the presence of Dex, interfering with protein phosphorylation, inhibits Bcl-2 expression and induces PCD in cells that are Bcl-2 negative.

In contrast to Bcl-2, we did not notice any difference in Bax and Bcl-x_L expression either during the progression of the cell cycle or in the presence of Dex. Recently, it has been shown that the two survival genes Bcl-2 and Bcl-x_L are differentially regulated by IL-2 and CD3 stimulation in memory T cells. While the Bcl-2 protein is strongly up-regulated in response to IL-2, the stimulation of the CD3 complex increased Bcl-x expression.⁴⁴ Moreover, resting helper T cell clones expressed no detectable Bcl-2 protein and a modest amount of Bcl-x protein. Our analysis of gene expression shows the same picture, demonstrating that Bcl-x_L is expressed with small differences in both resting and proliferating cells and the presence of Dex does not modify Bcl-x expression.

Another point that our study on apoptotic gene expression evidences is that FasL expression is detectable in proliferating cells and undetectable in cells arrested in the G0/G1 phase of the cell cycle. It is well known that FasL expression is induced in T cells following TCR triggering and that its expression is transient and quickly down-regulated.¹⁶ However, we noticed that once FasL is induced by antigen-stimulation, the addition of IL-2 sustains T cell proliferation and maintains FasL expression until they arrest. Moreover, our RT-PCR analysis reveals that Dex greatly reduces the expression of FasL and this result is in agreement with a previous observation by Young *et al.* on the ability of Dex to block FasL expression.⁴⁵

It is a common opinion that once the immune responses are activated, they must be carefully controlled or extinguished. In fact, antigen activated T cells produce copious amounts of inflammatory cytokines that contribute to induce hyperplasia in lymphonodes. It is therefore important that both cell number and cytokine synthesis are reduced when the antigen is eliminated. Glucocorticoids, able to influence both, have always been considered relevant modulators of immune response. This paper, underlining the failure of IL-2 to up-regulate bcl-2 expression in the presence of Dex, adds new information on the capacity of Dex to down-regulate immune responses when antigen concentration drops down and microenvironment cytokines are still available.

Materials and Methods

T cell clones

The T cell clones G12 and G3 were CD4⁺ DR1-specific alloreactive T cell clones derived from a DR4, DRw13 heterozygous individual as previously described.⁴⁶ The clones HC3 and HC6 were restricted by DR1 and specific for HA100-115 peptide and 307-319 respectively.⁴⁷ The clones were maintained in culture by weekly stimulation with irradiated (3000 rads) DR1-expressing PBMC in RPMI 1640 medium

with 10% human serum and 20 U/ml of human recombinant IL-2 (Boehringer Mannheim, Germany). HA100-115 peptide and 307-319 were added to HC3 and HC6 respectively.

DNA labeling technique and flow cytometric analysis of apoptosis

Apoptosis was measured by flow cytometry as previously described.⁴⁸ Briefly, T cell clones incubated with the tested substances were washed twice in PBS and stained by propidium iodide (PI) at 50 µg/ml following cell permeabilization in PBS plus 0.1% Triton X-100 and incubation at 37°C for 20 min. The DNA content was measured by a Becton-Dickinson FACStar plus flow-cytometer. Cells containing a DNA lower and a Side Scatter higher than that of G0/G1 cells were considered to be apoptotic cells. Data are representative of at least five independent experiments.

Indirect immunostaining

For intracellular Bcl-2 staining, T cells were fixed for 1 h on ice with 0.25% paraformaldehyde, washed, permeated for 15 min at 37°C with 0.2% Tween 20, washed twice in PBS and incubated with anti-Bcl-2 rabbit polyclonal antiserum (Boehringer Mannheim, Germany) for 40 min at 4°C. Finally, cells were washed twice in PBS and incubated with FITC-conjugated mouse-anti-rabbit (Sigma) for 40 min at 4°C. At the end of incubation, cells were washed and analyzed by a FACScan flow cytometer (Becton Dickinson).

Isolation of RNA and reverse transcription

Total RNA was isolated from cell pellets by the method of Chomczynski *et al.*⁴⁹ Briefly, cells were lysed by guanidium-isothiocyanate (4 M) in sodium citrate (25 mM) buffer, pH 7, with 0.5% sarcosyl and 0.1 M β2-ME. Subsequently, 0.1 vol of 2 M sodium acetate was added, together with 1 vol water-saturated phenol and 0.2 vol of chloroform: isoamyl alcohol (49:1). After centrifugation, RNA was precipitated with isopropanol and placed at -20°C for at least 1 h. Precipitated RNA was pelleted by centrifugation, washed with ethanol 70%, vacuum-dried and resuspended in DEPC-water. cDNA (corresponding to 2.5 × 10⁵ cells) was synthesized from oligo dT-primed RNA in 20 µl of reverse transcriptase buffer and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO, Bethesda Research Laboratories) incubated at 37°C for 1 h.

PCR amplification

PCR mixture containing the cDNA and 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM 5' and 3' oligonucleotide primers and 2.5 U Taq polymerase (Perkin Elmer, Cetus) was amplified in 0.2 ml Gene Amp tubes in a final volume of 50 µl. PCR reactions consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at the right temperature for each pair of primers and extension at 72°C for 30 s. Primer sequences used were: β-actin 5'-GTGGGGCGCCCCAGGCACCA, β-actin 3'-CTCCTTAATGT-CACGCACGATTTT (548 bp fragment); Fas 5'-GTACAGAAAACATG-CAGAAAGCAC, Fas 3'-CTCTGCAAGAGTACAAAGATTGGC (342 bp fragment); FasL 5'-CAAGTCCAACCTCAAGGTCCATGCC, FasL 3'-CAGAGAGAGCTCAGATACGTTGAC (356 bp fragment); Bcl-2 5'-GCGTCAACCGGGAGATGTCGCC, Bcl-2 3'-TTTCTTAAACAGCCTGCAGCTTTG (348 bp fragment); Bcl-x_L 5'-ATTGGT-GAGTCGGATCGCAGC, Bcl-x_L 3'-AGAGAAGGGGTGGGAGGGTA

(262 bp fragment) and Bax 5'-AGCTCTGAGCAGATCATGAAG, 3'-CTCCCGGAGGAAGTCCAATG (396 bp fragment). PCR products were size fractionated by agarose gel electrophoresis and normalized according to the amount of β-actin detected in the same mRNA sample. Photographs were taken with positive/negative 665 film (Polaroid Corp) of DNA bands visualized by ethidium bromide staining and UV transillumination.

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