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*J Immunol* 2006; 176:1695-1702; doi: 10.4049/jimmunol.176.3.1695
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Glucocorticoids Engage Different Signal Transduction Pathways to Induce Apoptosis in Thymocytes and Mature T Cells

Dapeng Wang, Nora Müller, Kirsty G. McPherson, and Holger M. Reichardt

Glucocorticoids (GC) induce apoptosis in a variety of cells, but their exact mode of action is controversial. Although initiation relies on the GC receptor (GR) and de novo gene expression, the effector phase differs among cell types. Proteasomal degradation as well as caspase-3, -8, and -9 activity are essential for GC-induced apoptosis in murine thymocytes, but the same enzymes are dispensable in splenic T cells. Live imaging by confocal microscopy revealed that lysosomal cathepsin B, an unrecognized component of this pathway to date, becomes rapidly activated in thymocytes after GC exposure. This is followed by leakage of cathepsin B into the cytosol, nuclear condensation, and processing of caspase-8 and -3. According to our model, activation of caspase-3 by caspase-9 in thymocytes occurs both directly as well as indirectly via a lysosomal amplification loop. Interestingly, acute T lymphoblastic leukemia cells depend on caspase activity to undergo GC-induced cell death similar to thymocytes. Collectively, the apoptotic program induced by GCs comprises cell type-specific as well as common features. The Journal of Immunology, 2006, 176: 1695–1702.

Glucocorticoids (GCs) are a class of steroid hormones that exert a wide range of anti-inflammatory, immunosuppressive, and antineoplastic activities, including the ability to induce apoptosis in T and B lymphocytes (1). This property of GCs is widely exploited to treat neoplastic disorders such as leukemia and lymphoma (1–3). However, despite being one of the first recognized forms of programmed cell death (4), the molecular mechanism of GC-induced apoptosis is still incompletely understood. GCs passively diffuse into the cell and bind to the GC receptor (GR), a member of the nuclear receptor superfamily (5). Subsequently, the hormone-receptor complex translocates into the nucleus, where it modulates gene expression either by direct binding to its cognate response elements or via interaction with other transcription factors. In the case of thymocyte apoptosis, it has been previously shown that gene activation is essential to this process (6). However, the genes required for initiating the cell death program are only beginning to be uncovered (7–11).

Concerning the effector phase of GC-induced apoptosis, many data suggest that it proceeds via the mitochondrial pathway involving Bcl-2 family members. Disruption of Bcl-2 in mice accelerates GC-induced thymocyte apoptosis, whereas lack of Bax and Bak prevents it (12, 13). In addition, Bim- and Puma-deficient mice are partially compromised in this process, supporting the idea that GCs may act by up-regulating proapoptotic BH3-only proteins (11, 14, 15). With regard to the involvement of caspases, gene-targeting experiments and pharmacological studies have arrived at conflicting results. Although the use of small peptide inhibitors has implicated caspase-3 and -8 in GC-mediated thymocyte apoptosis (16, 17), the respective knockout mice lack any obvious defects in this process (18, 19). In contrast, caspase-9 as well as Apaf-1-deficient mice are impaired in dexamethasone (Dex)-induced thymocyte cell death (20–22), whereas in vitro experiments are not in support of these findings (17, 23). Thus, although a large number of components have been implicated in GC-induced thymocyte apoptosis, the exact apoptotic program initiated by GCs remains controversial.

Besides the mitochondria, participation of the lysosome as well as the endoplasmatic reticulum in various apoptotic pathways is now well established (24). In particular, cathepsin B has gained acceptance as a central mediator of cell death. Cathepsins are synthesized as proenzymes, transported into the lysosomal vesicle, and activated through proteolytic cleavage (25). Although the controlled recycling of cellular macromolecules by cathepsins occurs within the lysosome, it has been observed that during certain forms of apoptosis, cathepsin B is also released into the cytosol. The mechanism of translocation is poorly understood, but there is evidence that it is achieved by caspases (25). Opening of pre-existing pores, active transport across the membrane, and limited lysosomal membrane rupture have all been hypothesized to account for the leakage of cathepsin B into the cytoplasm following apoptotic stimuli (26, 27). Once in the cytosol, cathepsin B can induce cell death by activating initiator caspases (26, 28) or directly cleaving nuclear substrates (26, 27). However, it is unknown whether one of these effector mechanisms is involved in GC-induced cell death.

One problem of the current discussion on the mechanisms of GC-induced apoptosis stems from the fact that different primary cells and lymphoma cell lines are compared irrespective of their individual cellular characteristics and gene expression profiles. Although it is generally assumed that GCs induce apoptosis via a conserved mechanism, this is not supported by any experimental data. We therefore wondered whether a unique signal transduction pathway is engaged by GCs to initiate and execute cell death in all...
types of T lymphocytes or whether distinct pathways exist. Our data now show that the initiation phase of GC-induced apoptosis is similar irrespective of the differentiation state of the cell. In contrast, execution of cell death in thymocytes and splenic T cells differs significantly in the requirement for signal transduction components such as the proteasome, caspases, and cathepsins. In the future, this could potentially form a basis for new anticancer strategies that specifically target tumor cells while leaving mature T cells of patients untouched.

Materials and Methods

Animal experimentation

All animals were kept in individually ventilated cages under specific pathogen-free conditions. GR knockout mice (29) backcrossed to C57BL/6 for >12 generations (GRN) and wild-type C57BL/6 mice (Charles River Laboratories) were bred in our own animal facility and used for the experiments at 6–12 wk of age. Because homozygous GRN+/− mice are not viable, heterozygous GRN+/− mice that express strongly reduced levels of GR protein were used in this study. All animal experiments were conducted in accordance with accepted standards of animal care and approved by the Bavarian state authorities.

Abs and reagents

The following Abs and reagents used for flow cytometry were obtained from BD Biosciences: annexin V FITC, annexin V Cy5, anti-mouse TCRβ Ab (H57-597), anti-Bcl-2 Ab, and active caspase-3 PE apoptosis kit. The CaspGLO Fluorescein Active Caspase-8 Staining Kit was purchased from BioVision. The anti-GR Ab was obtained from Santa Cruz Biotechnology, and the anti-β-tubulin polyclonal Ab, Dex, propidium iodide, cycloheximide, and mifpristone (RU486) were from Sigma-Aldrich. Caspase-3 and -8 inhibitors were purchased from R&D Systems, and lactacystin, pan-cathepsin inhibitor, cathepsin B inhibitor, and PD150606 are from Calbiochem.

Cell isolation and culture

Thymocytes and spleen cells were isolated by passing the freshly isolated organs through a nylon mesh, followed by repeated washes with PBS. Splenic T cells were purified by MACS (Miltenyi Biotec), according to the manufacturer’s instructions. In brief, spleen cells were stained with FITC-conjugated H57 Ab, followed by incubation with anti-FITC-coupled magnetic microbeads (Miltenyi Biotec). The labeled cells were passed through an LS column positioned in a magnetic field, and the T cell fraction was subsequently eluted. Purity was assessed by flow cytometry and routinely found to be >95%. After counting, thymocytes and splenic T cells were resuspended in RPMI 1640 medium containing 10% FCS and standard serum complements (Invitrogen Life Technologies) at a concentration of 10^6 cells/ml and cultured in 96-well plates at 37°C in an incubator (Fig. 1C). Taken together, these results suggest that initiation of GC-mediated apoptosis depends on GR-induced de novo gene expression irrespective of the differentiation stage of the T cell.

Cell survival

Thymocytes and splenic T cells were cultured in 96-well plates at 37°C in an incubator supplemented with 20 mM HEPES buffer, and added to the coated chambers. Data were collected for 1.5 h at 5-min intervals. The temperature of the sample was maintained at 37°C using an objective heater.

Statistical analysis

All data were analyzed using the program Statistica. Comparison of two experimental groups was achieved using Student’s t test. For comparison of several groups, a one-way ANOVA followed by a posthoc Tukey honest significant difference test was performed. The statistical significance of selected comparisons is depicted in the figures. *p < 0.05; **p < 0.01; ***p < 0.001.

Results

GC-induced apoptosis of T lymphocytes is mediated by the GR and requires de novo gene expression

It has previously been shown that GC-induced thymocyte apoptosis involves gene activation by the GR (6). However, the mechanism underlying initiation of apoptosis in mature T lymphocytes is not clear. Therefore, we first investigated GC-induced apoptosis in thymocytes and splenic T cells from heterozygous GRN+/− knockout mice (29), in which levels of GR are greatly reduced (data not shown). The dose-response curve of Dex-induced apoptosis in GRN+/− cells was shifted to higher hormone concentrations in both cell types as compared with GR+/+ control cells, indicating that the presence of the GR was essential (Fig. 1A). To investigate whether altered gene expression underlies GC-induced apoptosis, we used the pharmacological inhibitor RU486, which is known to block the agonistic activity of the GR by competitive binding to the ligand-binding domain (30). As expected, RU486 prevented GC-induced apoptosis in thymocytes and splenic T cells (Fig. 1B). Finally, we investigated the need for de novo gene expression using cycloheximide. This protein synthesis inhibitor completely prevented Dex-induced apoptosis in thymocytes and splenic T cells within the first 10 h after addition of the hormone (Fig. 1C). Taken together, these results suggest that initiation of GC-mediated apoptosis depends on GR-induced de novo gene expression irrespective of the differentiation stage of the T cell.

Caspase activity is essential for GC-induced apoptosis of thymocytes, but not splenic T cells

Previous experiments have implicated caspase-3, -8, and -9 in GC-induced thymocyte apoptosis, but their role in mature T cells has not been explored (16, 17). Therefore, we treated thymocytes and splenic T cells with 10−7 M Dex for 20 h in the absence or presence of the pan-caspase inhibitor Z-VA-D-FMK or inhibitors specific for caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), or caspase-9 (Z-LEHD-FMK). Apoptosis was subsequently determined by flow cytometry using annexin V FITC and propidium iodide. In thymocytes, the pan-caspase inhibitor completely prevented Dex-induced apoptosis. Inhibition of caspase-3, -8, and -9

SDS-PAGE gel, transferred onto a polyvinylidene difluoride membrane, and stained with the indicated Abs. All secondary Abs were peroxidase coupled, and the blots were developed using ECL as a substrate (Amerham). Normalization was achieved by staining with Abs against β-tubulin or p56Xk.

Microscopic detection of cathepsin B activity in living cells

Imaging was performed using a Zeiss LSM 410 confocal microscope and a ×63 oil objective. A total of 1 × 10^5 cells per sample of freshly isolated thymocytes or splenic T cells was treated for 1 h with Dex or PBS, and subsequently incubated for 30 min with the cathepsin B substrate, a (z-Arg-Arg)-derivative of the cresyl violet fluorophore (Metachem). After incubation, the cells were washed, resuspended in PBS, and immediately observed under the confocal microscope. At least 200 cells were counted for each treatment. For live cell imaging, eight-chamber coverslides (LAB-TEK II; Nunc) were coated with 0.01% poly-l-lysine (Sigma-Aldrich) for 5 min, extensively washed with PBS, and dried. After incubation with the cathepsin B substrate, the cells were resuspended in complete RPMI 1640 medium, supplemented with 20 mM HEPES buffer, and added to the coated chambers. Data were collected for 1.5 h at 5-min intervals. The temperature of the sample was maintained at 37°C using an objective heater.
also significantly reduced the extent of cell death, although the effects were incomplete (Fig. 2A). In contrast, none of the caspase inhibitors was able to prevent Dex-induced apoptosis in splenic T cells (Fig. 2A). The presence of the inhibitors alone had no effect on cell survival (data not shown). A similar difference between thymocytes and mature T cells was also found in rat cells, confirming that our observations are not species specific (data not shown).

Cleavage of caspase-3 into its active form is an essential step of most forms of apoptosis (31). To obtain further evidence for the observed cell type specificity, we investigated cleavage of caspase-3 after GC treatment by Western blot and flow cytometry. Thymocytes and splenic T cells were cultured for 8 h in the presence or absence of 10^{-7} M Dex, and the time course of cleavage was studied by Western blot. A band corresponding to the processed enzyme was detected in Dex-treated thymocytes as early as 2 h, but at no time in splenic T cells (Fig. 2B). This result was confirmed on a single cell basis by flow cytometry taking

FIGURE 1. The execution phase of GC-induced apoptosis in thymocytes and splenic T cells is similar. A. Thymocytes and splenic T cells from wild-type (GRN^{+/+}) and heterozygous GR knockout mice (GRN^{+/−}) were cultured in the presence of Dex for 20 h. Relative survival compared with the untreated control culture was determined by annexin V/PI staining. n = 5 for thymocytes; n = 3 for splenic T cells. B. Thymocytes and splenic T cells were treated with 10^{-7} M Dex in the absence (D) or presence (D/R) of 10^{-6} M RU486 for 20 h, and the relative survival was determined at the 10- and 20-h time points. Survival in the untreated control culture (con) was set at 100%. C. Thymocytes and splenic T cells were treated with 10^{-7} M Dex in the presence or absence of 10 μM cycloheximide for 10 h. Every 2 h, the relative survival as compared with the untreated control culture was determined. The error bars represent the SEM for three independent experiments with three replicates each. Statistical significance was determined using Student’s t test, as described in Materials and Methods.

FIGURE 2. Caspase activity is essential for GC-induced thymocyte, but not splenic T cell apoptosis. A. Thymocytes and splenic T cells were treated with 10^{-7} M Dex in the absence or presence of 100 μM pan-caspase (D/P), caspase-3 (D/3), caspase-8 (D/8), or caspase-9 (D/9) inhibitor for 20 h. Relative survival was determined by annexin V/PI staining and set as 100% for controls. The error bars represent the SEM for three independent experiments with three replicates each. Statistical significance was determined by ANOVA, as described in Materials and Methods, and is solely depicted for the comparison of individual inhibitor treatments with the Dex-treated cells. B. Thymocytes and splenic T cells were cultured in the absence or presence of 10^{-7} M Dex for 8 h. Cells were collected at 2-h intervals, and the proteins were extracted. Full-length caspase-3 (C3) and its cleaved form were visualized by Western blot; staining for Lck served as a loading control. One representative experiment of three is depicted. C, Thymocytes and splenic T cells were treated with 10^{-7} M Dex or PBS (con) for 10 h, and the percentage of cells in the live gate that positively stained for cleaved (active) caspase-3 was determined by flow cytometry using a PE-conjugated mAb. One representative FACS analysis is depicted for each sample. The percentages refer to the proportion of cells containing active caspase-3. D, Time course of caspase-3 cleavage in thymocytes (Thy) and splenic T cells (Tc) treated with 10^{-7} M Dex or PBS measured by flow cytometry as in C. E, Thymocytes were treated with Dex in the presence or absence of 100 μM caspase-3 (D/3), caspase-8 (D/8), or caspase-9 (D/9) inhibitor for 10 h, and the percentage of live cells containing active caspase-3 was determined by flow cytometry. The error bars in D and E represent the SEM for three independent experiments with three replicates each. Analysis in E was achieved by ANOVA, as described in Materials and Methods; statistical significance is only depicted for the comparison of individual inhibitor treatments with the Dex-treated cells.
advantage of an Ab specifically detecting the cleaved form of caspase-3. Dex treatment led to an activation of caspase-3 in ∼80% of all thymocytes within the first 10 h (Fig. 2, C and D). In contrast, no cleavage of caspase-3 was observed in Dex-treated splenic T cells, even after prolonged incubation for 24 h (Fig. 2, C and D, and data not shown). Importantly, cleavage could be prevented by inhibitors of caspase-3, -8, and -9, suggesting that caspase-3 is positioned downstream of the other two caspases (Fig. 2E). Taken together, these results suggest that thymocytes and mature T cells significantly differ in their requirement for caspase activity to undergo apoptosis after GC treatment.

Proteasomal degradation and cathepsin activity are required for GC-induced apoptosis of thymocytes, but not splenic T cells

It was previously shown that GC-induced apoptosis in thymocytes involves proteasomal degradation (32, 33). Given that caspase activity is dispensable for GC-induced apoptosis of mature T cells, we wondered whether this was also true for proteasomal processes. To this end, we treated thymocytes and splenic T cells with 10⁻⁷ M Dex in the absence or presence of lactacystin and followed apoptosis over the first 10 h (Fig. 3). As expected, lactacystin efficiently prevented cell death in thymocytes (33). However, the same compound had no effect on GC-induced apoptosis in splenic T cells. Similar results were obtained with the proteasome inhibitor MG-115 (data not shown), confirming that proteasome activity was indeed dispensable for GC-induced apoptosis in mature T cells.

Recently, lysosomal proteases from the family of cathepsins have been implicated in various forms of programmed cell death (25, 27, 34). We therefore tested their requirement for GC-induced apoptosis in thymocytes and splenic T cells (Fig. 3). The pan-cathepsin inhibitor Z-FG-NHO-Bz and the cathepsin B inhibitor Z-FA-CH₂F (35) both significantly reduced the extent of Dex-induced apoptosis in thymocytes. In contrast, the same inhibitors were only weakly effective in splenic T cells. Importantly, the cathepsin inhibitors are known not to cross-react with caspases (16, 36, 37). For comparison, the calpain inhibitor PD150606, which blocks nonlysosomal cysteine proteases, had no effect on GC-induced apoptosis in either cell type (Fig. 3). Taken together, these results reveal for the first time a role for cathepsin activation in GC-induced thymocyte apoptosis.

Cathepsin B rapidly becomes activated after GC exposure and is part of a lysosomal amplification loop

Based on the inhibitor studies, cathepsin B plays an essential role in GC-induced cell death in thymocytes. To confirm this finding, we studied cathepsin B activation by confocal microscopy using a substrate that is converted into a fluorescent product by the active enzyme (34). Within 90 min after Dex exposure, about one-half of all thymocytes stained positive for active cathepsin B, whereas this was only rarely seen in control cultures (Fig. 4, A and B). In contrast, active cathepsin B was found in many splenic T cells directly after isolation, but, importantly, there was no change observed after addition of Dex for up to 6 h (Fig. 4A and data not shown). In keeping with this finding, enumeration of cells containing active cathepsin B revealed a significant difference between Dex- and PBS-treated thymocytes, but not splenic T cells (Fig. 4B and data not shown). Staining with a green fluorescent lysotracker further suggests that cathepsin B activation indeed occurs in lysosomes as expected (data not shown). To study the position of cathepsin B in the apoptotic pathway relative to the involved caspases, we studied its activation in the presence of various pharmacological inhibitors by confocal microscopy. Cathepsin B inhibitor prevented the accumulation of fluorescent cells, while caspase-3 and -8 inhibitor did not have any significant effect (Fig. 4C). This suggests that cathepsin B activation precedes the activation of caspase-3 and -8. In contrast, inhibition of caspase-9 completely abolished cathepsin B activity, indicating that the lysosome acts downstream of caspase-9. Given the known ability of caspase-9 to directly activate caspase-3, we hypothesize that the lysosomal program forms an amplification loop that functions in parallel to the direct effects of the apoptosome.

Finally, we investigated the kinetics of Dex-induced thymocyte apoptosis by live imaging of cathepsin B activity using confocal microscopy. Single cells were selected and observed over a period of 75 min starting 90 min after GC treatment (Fig. 4D). Within 15 min, strongly fluorescent dots accumulated within the cell, representing active cathepsin B. After 30 min, the active enzyme was released from the lysosomes, the fluorescence intensity increased,
were treated with 10^{-7} M Dex for 1 h. Subsequently, the cells were incubated with the substrate for 30 min, and cathepsin B activity was observed by confocal microscopy. One representative experiment of three is depicted. **B**, Enumeration of thymocytes positively staining for active cathepsin B, as shown in A. The error bars represent analysis of \( \geq 200 \) representative cells each. **C**, Thymocytes were treated with 10^{-7} M Dex in the presence or absence of inhibitors of cathepsin B (CatB), caspase-3 (C3), caspase-8 (C8), or caspase-9 (C9) for 1 h. Treatment with the vehicle alone (0.2% DMSO) served as a control. Cells positively staining for active cathepsin B were determined and enumerated, as described for **B**. Analysis was achieved by ANOVA, as described in **Materials and Methods**. Statistical significance is only depicted for the comparison of individual inhibitor treatments with the Dex-treated cells. **D**, Thymocytes were treated with 10^{-7} M Dex for 1 h, followed by incubation with the substrate for 30 min. Subsequently, cathepsin B activity was observed in single cells at 5-min intervals for the following 75 min. The kinetics and distribution of cathepsin B activity for a representative thymocyte are depicted. The observed cell is marked by an open arrow. The bar equals 5 \( \mu \)m in all panels.

Proteasomal degradation precedes caspase-8 activation

Our data indicate that caspase-8 activation occurs after cathepsin B has been released into the cytosol, but before caspase-3 cleavage. To obtain independent evidence for this sequence of events, we directly studied activation of caspase-8 by flow cytometry using the cell-permeable, nontoxic substrate FITC-IETD-FMK, which irreversibly binds to the activated enzyme. Thymocytes and splenic T cells were cultured in the presence or absence of 10^{-7} M Dex for 10 h, and the percentage of live cells containing active caspase-8 was determined. As already suggested by the inhibitor studies, caspase-8 was exclusively activated in thymocytes, but not in splenic T cells following GC treatment (Fig. 5, A–D). Culture of thymocytes in the presence of caspase-8 inhibitor completely prevented its activation, as did caspase-9 inhibitor, cathepsin B inhibitor, and lactacystin (Fig. 5 C). These results confirm the previously made observation that cathepsin B precedes caspase-8 activation (Fig. 4 C), and at the same time place the proteasome and caspase-9 upstream of caspase-8. In support of this notion, both

**FIGURE 4.** Cathepsin B activation is an early event during GC-induced thymocyte apoptosis. **A**, Thymocytes and splenic T cells were cultured in the presence (Dex) or absence (PBS) of 10^{-7} M Dex for 1 h. Subsequently, nuclear condensation was observed. Then, the enzyme activity became confined to the contracting remnants of the cell until it was finally almost undetectable (Fig. 4 D). Importantly, a similar release of cathepsin B into the cytosol after Dex exposure was never observed in mature T cells, confirming that the involvement of the lysosomal apoptotic pathway is restricted to thymocytes (data not shown).

**FIGURE 5.** Caspase-8 activation occurs downstream of proteasomal degradation and lysosomal processes. **A** and **B**, Thymocytes and splenic T cells were treated with 10^{-7} M Dex or PBS (con) for 10 h, and the percentage of cells in the live gate that stained positively for activated caspase-8 was determined by flow cytometry. One representative FACS analysis is depicted each. The percentages refer to the proportion of cells containing active caspase-8. **C**, Caspase-8 activation was determined in thymocytes treated with Dex in the presence or absence of 100 \( \mu \)M caspase-8 (D/8), caspase-9 (D/9), cathepsin B inhibitor (D/B), or 8 \( \mu \)M lactacystin (D/L), as described for A. **D**, Caspase-8 activation was determined in splenic T cells cultured in the presence or absence of 10^{-7} M Dex, as described for B. **E**, Thymocytes were treated with Dex in the presence or absence of 100 \( \mu \)M cathepsin B inhibitor (D/B) or 8 \( \mu \)M lactacystin (D/L), and the percentage of live cells containing active caspase-3 was determined by flow cytometry, as described for Fig. 2. The error bars in C–E represent the SEM for three independent experiments with three replicates each. Analysis was achieved by ANOVA, as described in **Materials and Methods**. Statistical significance is only depicted for the comparison of individual inhibitor treatments with the Dex-treated cells.
The apoptotic program induced by GCs in lymphoid tumor cells partially resembles the one in murine thymocytes

Induction of lymphocyte apoptosis is the therapeutic principle underlying the treatment of lymphoproliferative malignancies such as lymphoma and leukemia with synthetic GCs (3). To investigate the signal transduction pathway engaged by GCs in lymphoid tumor cells, we studied three different acute T lymphoblastic leukemia (T-ALL) cell lines. Jurkat J.Gr cells are a derivative of the human cell line Jurkat E6.1, which had been transduced with a GR-encoding lentivirus (van den Brandt et al., submitted for publication). TALL-1 is also of human origin and has previously been shown to respond to GC treatment with growth arrest (38). WEHI7.1 is a mouse T-ALL cell line frequently used to study GC-induced apoptosis (11, 39, 40).

First, we studied GR protein levels in all three lines. Whereas expression in J.Gr and TALL-1 cells was comparable to murine thymocytes, the level of GR protein in WEHI 7.1 cells was much lower (Fig. 6A). Furthermore, TALL-1 cells express high levels of Bcl-2, which was undetectable in both WEHI 7.1 and J.Gr cells (Fig. 6A). These findings may explain the comparably high resistance of TALL-1 and WEHI7.1 cells to GC-induced apoptosis, i.e., both lines require treatment with $10^{-5}$ M Dex to achieve a decent level of apoptosis, whereas in the case of J.Gr cells $10^{-7}$ M Dex is sufficient.

FIGURE 6. The death signal-transducing pathway in T-ALL cells resembles the one in thymocytes. A, Comparative analysis of GR and Bcl-2 protein levels in Jurkat J.Gr, TALL-1, and WEHI7.1 cells and murine thymocytes by Western blot. Staining with an anti-$\beta$-tubulin Ab served as a loading control. B, J.Gr cells were treated for 24 h with $10^{-7}$ M Dex in the presence or absence of 10 $\mu$M pan-caspase (D/P), caspase-3 (D/3), caspase-8 (D/8), caspase-9 inhibitor (D/9), or vehicle (0.1% DMSO, con). Relative survival was determined by annexin V/PI staining. C, and D, TALL-1 cells were treated for 72 h and WEHI7.1 cells for 24 h with $10^{-3}$ M Dex in the presence or absence of the same caspase inhibitors as in B, and the relative survival was determined. The error bars represent the SEM for three independent experiments with three replicates each. Analysis was achieved by ANOVA, as described in Materials and Methods. Statistical significance is only depicted for the comparison of individual inhibitor treatments with the Dex-treated cells.

To investigate whether GC-induced lymphoid tumor cell death shares characteristics of thymocyte or splenic T cell apoptosis, we exposed them to Dex in the presence or absence of caspase inhibitors. Similar to murine thymocytes, cotreatment of all three cell lines with any of the caspase inhibitors fully prevented induction of apoptosis (Fig. 6, B–D). Interestingly, the inhibitors were already effective at 10 $\mu$M, suggesting that the cell lines are more sensitive to inhibition of caspases than primary lymphocytes. Unfortunately, the effects of cycloheximide, lactacystin, and cathepsin B inhibitor could not be assessed because they turned out to be toxic already at very low concentrations (data not shown). Taken together, it appears that, at least with regard to the involvement of caspases, the apoptotic program induced by GCs in T-ALL cell lines resembles the one in thymocytes.

Discussion

More than a quarter of a century ago, the phenomenon of GC-induced lymphocyte apoptosis was first recognized (4), but our knowledge about the signal transduction pathways engaged by GCs in this process is still incomplete (1–3). Gene-targeting and inhibitor studies have implicated GR-mediated gene activation, pro- and antiapoptotic Bcl-2 family members, as well as caspases in GC-induced cell death, but to date these components have not been assembled in a cohesively scheme. Little attention has also been given to the cell type specificity of GC-induced apoptosis. Most investigators in the past have focused on analyzing apoptosis in thymocytes, although it has been shown that the pan-caspase inhibitor Z-VAD-FMK fails to prevent Dex-induced cell death of mature, peripheral T cells (41). The relevant knockout mouse models such as caspase-3 and -9 as well as Apaf-1-deficient mice have been exclusively analyzed for Dex-induced apoptosis of thymocytes, but not mature T lymphocytes (19–22).

Although GC-induced cell death has been studied in peripheral T cells of conditional caspase-8 knockout mice (18), this was only done using activated cells known to be partially protected from GC action. Thus, a systematic comparison of different cell types for their sensitivity to GCs has not been performed to our knowledge. We have now found that the execution phase of GC-induced apoptosis differs between thymocytes and splenic T cells. Several components that are critical to this process were identified in thymocytes, whereas none of them seem to play a role in GC-induced apoptosis of splenic T cells. Importantly, we could define a set of events exclusively occurring in thymocytes. These include activation of caspase-3, -8, and -9, proteasomal degradation, and the requirement for a lysosomal pathway involving cathepsin B. This shows for the first time that GCs induce distinct apoptotic programs in thymocytes and mature T cells (Fig. 7). However, whereas a vast amount of data is available concerning GC-induced thymocyte apoptosis, we still have no clue as to the pathway used by GCs to induce cell death in peripheral T cells. Besides the analyses described in this study, further attempts to demonstrate a role for serine proteases or the apoptosis-inducing factor, a mediator of caspase-independent cell death (42), failed (data not shown).

Recent work from several laboratories has identified a number of genes, such as T-DAG8, dig-2, Bim, and PUMA, which become activated during GC-induced apoptosis. Although the importance of these proteins was confirmed by the analysis of knockout and transgenic mice, RNA interference, and pharmacological approaches, their exact role in the initiation and execution of the apoptotic program induced by GCs remains elusive (7–9, 11, 14, 43, 44). The role of caspases is also contentious. Caspase-3, -8, and -9 have been ascribed a role in GC-induced thymocyte apoptosis by some investigators, but not by others (17–23, 45). Attempts to solve this dilemma include the postulate of bifurcated pathways.
redistribution into the cytosol, in which cell death is initiated either
becomes activated and subsequently released (25). This leads to its
general model of lysosomal cell death suggests that cathepsin B
some at an early stage of GC-induced thymocyte apoptosis. The
effect is unlikely to be important (48). We have now defined an
the generally fast apoptotic response to this stimulus, the observed
tion of cathepsin B in GC-mediated cell death remained largely
amplification loop. This finally results in cell death, as evident by phos-
phatidylserine exposure, membrane permeabilization, and cell shrinkage.
The pathway used in mature T cells remains elusive.

Lepine et al. (23), for example, suggested a model in which
caspase-8 is activated in parallel to caspase-9, with both converging
on caspase-3. Although our concept of GC-induced thymocyte
apoptosis differs a little from their model (see below), it also as-
sumes branched pathways and suggests an amplification loop anal-
ogous to that shown for death receptor-induced apoptosis in type II
cells (46).

A novel aspect of this study is the identification of cathepsin B
activation being an early and essential step in the execution phase
of GC-induced thymocyte apoptosis. It was previously shown that
lysosomes and cathepsin B play an important role in the induction
of apoptosis in a variety of cell types (24, 47). However, the func-
tion of cathepsin B in GC-mediated cell death remained largely
unexplored. One study has demonstrated increased cathepsin B
mRNA levels 24 h after Dexam treatment in thymocytes, but given
the generally fast apoptotic response to this stimulus, the observed
effect is unlikely to be important (48). We have now defined an
important role for caspase B activity and consequently the lys-
some at an early stage of GC-induced thymocyte apoptosis. The
general model of lysosomal cell death suggests that cathepsin B
becomes activated and subsequently released (25). This leads to its
redistribution into the cytosol, in which cell death is initiated either
through caspase activation or direct cleavage of nuclear substrates
(26, 27). In the case of TNF-α-mediated hepatocyte apoptosis, this
establishes an amplification loop to enhance cell death (25). Our
findings on GC-induced apoptosis in thymocytes agree with this
model. In summary, we propose the following pathway for GC-
induced apoptosis of thymocytes (Fig. 7). Initiation of the apopto-
tic program proceeds via the mitochondria and results in caspase-9
activation. This directly leads to caspase-3 cleavage through the
apoptosome. However, in contrast to other forms of apoptosis, a
lysosomal amplification loop is required. This involves activation
and cytosomal leakage of cathepsin B, followed by caspase-8 ac-
tivation, and also converges on caspase-3. Only when the two
pathways act in concert, caspase-3 is sufficiently activated to ini-
tiate cell death.

T lymphocyte apoptosis induced by synthetic GC derivatives is
an important component of therapeutic protocols used to treat vari-
ous forms of lymphoma and leukemia. Because under these reg-
imens not only the neoplastic cells, but also the remaining healthy
lymphocytes are affected, strategies would be desirable that allow
the transformed cells to be targeted more selectively. In this con-
text, it is interesting that apoptosis induced in three T-ALL cell
lines shares its dependence on caspase activity with thymocytes,
but not mature T cells. Although involvement of other components
such as the proteasome and cathepsin B could not be addressed in
this study due to the toxicity of the inhibitors, it still suggests that
parallels exist in the apoptotic program induced by GCs in thym-
cytes and T-ALL cells. From this, one would expect that ad-
ressing components of the signal transduction pathways used
only by thymocytes and lymphoma cells, but not mature peripheral
T cells may represent a possible strategy for a more selective can-
ter therapy. Although suitable candidates have not yet been iden-
tified in this work, our results feed the hope that such a concept
might work and be developed in the future.

Acknowledgments

We thank Marco Heroit for critical reading of the manuscript,
Melanie Schott and Katrin Voss for excellent technical help,
Thomas Hınıg for fruitful scientific discussions, Denise Tischner for ad-
vice with the statistical analyses, and François Tronche for providing the
GRN mice.

Disclosures

The authors have no financial conflict of interest.

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FIGURE 7. Working model of GC-induced apoptosis in thymocytes.
GCs induce cell death via binding to the GR, followed by induction of de
novo gene expression irrespective of the cell type. In thymocytes, this leads
to the activation of caspase-9, which either directly cleaves caspase-3 or
activates cathepsin B within the lysosomes. This is followed by leakage
into the cytosol, activation of caspase-8 and caspase-3, thus establishing an
amplification loop. This finally results in cell death, as evident by phos-
phatidylserine exposure, membrane permeabilization, and cell shrinkage.