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Review

Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance

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Abstract

The ability of glucocorticoids (GC) to efficiently kill lymphoid cells has led to their inclusion in essentially all chemotherapy protocols for lymphoid malignancies. This review summarizes recent findings related to the molecular basis of GC-induced apoptosis and GC resistance, and discusses their potential clinical implications. Accumulating evidence suggests that GC may induce cell death via different pathways resulting in apoptotic or necrotic morphologies, depending on the availability/responsiveness of the apoptotic machinery. The former might result from regulation of typical apoptosis genes such as members of the Bcl-2 family, the latter from detrimental GC effects on essential cellular functions possibly perpetuated by GC receptor (GR) autoinduction. Although other possibilities exist, GC resistance might frequently result from defective GR expression, perhaps the most efficient means to target multiple antileukemic GC effects. Numerous novel drug combinations are currently being tested to prevent resistance and improve GC efficacy in the therapy of lymphoid malignancies.
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Abbreviations: ALL, acute lymphoblastic leukemia; GC, glucocorticoid(s); GR, glucocorticoid receptor

Introduction

Glucocorticoid (GC)-induced apoptosis is a phenomenon of considerable biological and clinical significance. Biologically, it has been implicated in the generation of the immune repertoire and the regulation of immune responses,[1–3] and clinically it has been exploited in the therapy of lymphoid malignancies.[4] In this review, we summarize current concepts regarding the molecular mechanism of this GC response and resistance against it, and discuss the potential clinical impact of emerging knowledge in this field. Space limitations precluded a complete reference to the large body of literature and we apologize to our colleagues for often citing reviews and exemplary work rather than all relevant original publications. To put our topic into perspective, we first provide a short introduction to the multitude of GC effects and their basic mechanism of action, and briefly discuss distinct forms of cell death as they relate to the topic of this review. The subsequent section summarizes the molecular components of GC-triggered death pathway, beginning with the role of glucocorticoid receptor (GR) expression, and subsequently addressing the controversial question of whether transactivation or transrepression is required, outlining GC-regulated genes as revealed by gene expression profiling studies, and finally providing a tentative model for this death response. The next section on Mechanisms of resistance to GC-induced apoptosis deals with GC resistance with particular emphasis on mechanisms acting at the level of the GR. The clinical significance of these phenomena and issues related to exploiting the true therapeutic potential of GC in novel combination protocols are topics of the last section.

Pleiomorphic effects of GC

Depending on a number of modulating factors, such as GC type and concentration, extracellular milieu, intracellular context, etc, GC and their analogues mediate a variety of effects on mammalian cells and entire organisms. These include pronounced effects on metabolism that primarily lead to catabolism of proteins, lipids and carbohydrates. GC increase blood sugar levels, cause osteoporosis, and play an important role in the stress response. They further repress cell cycle progression in a number of systems including acute lymphoblastic leukemia (ALL).[5,6] At least in therapeutic concentrations, GC are strongly immunosuppressive and anti-inflammatory,[7] which has made them one of the most frequently prescribed drugs worldwide.

Pertinent to this review, GC influence survival in many tissues in a cell-type-specific manner. As documented by over 2200 publications in the PubMed database and summarized in numerous recent reviews,[5,8–13] GC induce massive apoptosis in certain cells of the lymphoid lineage, particularly immature thymocytes and ALL cells, and the latter has been exploited in the therapy of lymphoid malignancies.[4] GC have further been reported to induce cell death (alone or in

combination with other death inducers) in some nonlymphoid tissues and cells such as bone,[14] hippocampus,[15] eosinophils,[16] fibroblasts[17] and certain cancer cells.[18] Interestingly, GC support survival in erythroblasts,[19] neutrophils[20] and several nonhematologic tissues such as mammary gland, ovary, liver and fibroblasts (reviewed in Amsterdam and Sasson[21]). Such prosurvival effects may become clinically relevant when they interfere with the effect of chemotherapeutics.[22] Depending upon the circumstances, GC both triggered cell death and supported survival in some cells,[23] further documenting the pro- and antiapoptotic potential of this hormone.

General mode of action of GC

Although receptor-independent effects may occur at very high concentrations (presumably through membrane perturbation[24]), most, if not all, effects of GC at physiologic or therapeutic levels are mediated by the GC receptor (GR, Figure 1). The GR is a ligand-activated transcription factor of the nuclear receptor family (steroid receptor subfamily, comprised of seven members: estrogen receptor α and β, estrogen-related receptors 1 and 2, and the receptors for mineralcorticoids, androgens and progesterone).[25] It resides in the cytoplasm in a multiprotein complex.[26] Upon ligand binding, the GR dissociates from at least some of its binding proteins and translocates into the nucleus to induce or repress the expression of a plethora of genes identified by conventional gene searches (reviewed in Geley *et al.*)[27] or microarray analyses.[28–38] Gene induction is mediated via GR interaction with conserved response DNA elements (GC responsive elements, GREs: GGTACANNNTGTTCT[25]), whereas gene repression occurs through negative GREs, protein–protein interaction with other sequence-specific transcription factors, competition for coactivators and other mechanisms (reviewed in Laudet and Gronemeyer[25] and Geley *et al.*[27]). While this is probably the mechanism underlying most GC effects, these hormones can exert more immediate (20–30 min), presumably nongenomic but still GR-dependent, effects, the mechanism of which is less well understood.[24,39] In addition to the well-characterized cytoplasmic/nuclear GR, a membrane-associated species was reported,[40] but its existence and possible significance remained controversial.

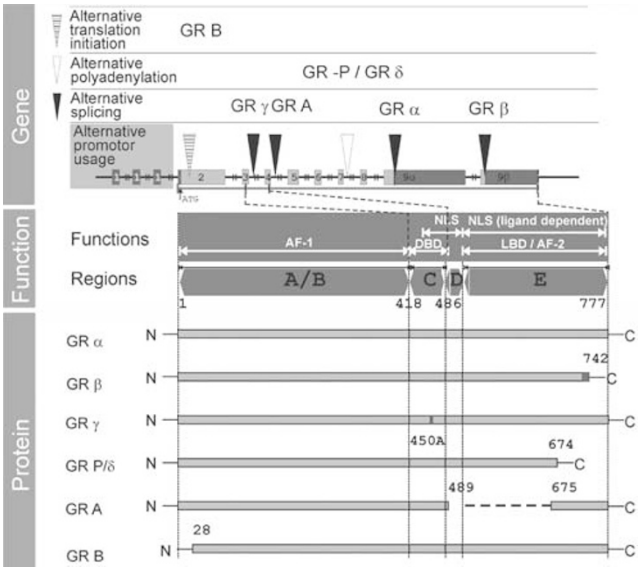

Figure 1 The human GR gene and the known GR variants. The top panel summarizes the genomic organization of the GR gene (NR3C1) on chromosome 5q31/32 and depicts various molecular mechanisms leading to six variant GR transcripts. Their schematic protein structure is given in the bottom panel. The middle panel relates the intron/exon structure and protein regions to their presumed function. Note that the GR gene has five published[125] and at least four additional untranslated exons 1 (Presul *et al.*, in preparation) of unknown significance. a/b, c, d and e refer to protein regions of nuclear receptors, the numbers denote amino-acid positions.[25] AF, sequences implied in transactivation; DBD, DNA-binding doman; LBD, ligand-binding domain; NLS, nuclear localization sequence

Cell death forms: apoptosis, necrosis and the 'in betweens'

Classically, two major cell death forms have been distinguished: (1) apoptosis, an active and ordered form of cellular suicide characterized by a number of morphologic criteria such as cell shrinkage, membrane blebbing, formation of apoptotic bodies, DNA cleavage and condensation, caspase activation, phosphatidylserine expression on the outer cell membrane, etc., and (2) necrosis or accidental cell death with membrane rupture and subsequent release of potentially inflammatory cell constituents into the surrounding tissue. Regarding apoptosis, two major signaling pathways have been described: the 'extrinsic' pathway that is initiated by ligand-mediated activation of membrane death receptors, and the 'intrinsic' pathway that is controlled by members of the Bcl-2 family and mitochondria-derived proteins. In the context of this review, we suggest to further differentiate between two conceptually distinct types of apoptotic cell death (Figure 2). In the first, apoptosis occurs in an entirely healthy cell because the apoptosis machinery has been activated (e.g., by specific

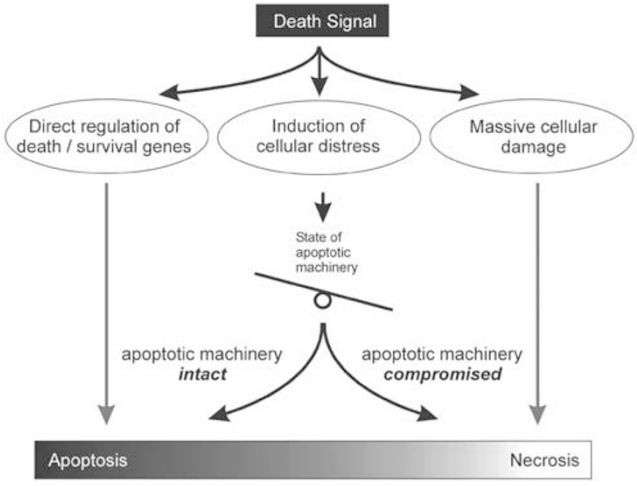

Figure 2 Hypothetical classification of cell death forms. In this model, death signals induce cellular demise in three different ways: first, by directly regulating crucial death or survival genes leading to apoptotic cell death; second, through cellular distress that might lead to either apoptosis or necrosis depending upon the availability of the apoptotic machinery; third, via massive cellular damage leading to necrotic cell death (for other details see text)

regulation of its key components). In the second, the apoptosis machinery is activated because the cell recognizes, and responds to, a harmful and potentially deadly insult. In the first, viability can be restored by interference with apoptosis effectors, which is not the case in the second, because blocking apoptosis does not affect the primary, and ultimately deadly, insult. In such instances, that is, when the cell is damaged but the apoptotic machinery is completely or partially compromised, the resulting cell death may adopt a more or less necrotic morphology. As discussed below in more detail, GC may induce cell death via several of these mechanisms.

Molecular mechanisms of GC-induced apoptosis

Initiation of the apoptotic response: role of the GR and its regulation

GC-induced apoptosis is initiated by, and strictly dependent upon, the interaction of GC with its receptor, the GR. The requirement for the receptor has been shown in thymocytes from genetically modified mice[41] and human ALL cell lines[42] with mutated GR, and by conferring GC sensitivity to GC-resistant ALL cell lines by GR transgenesis.[43,44] Moreover, the level of GR expression is a critical determinant for GC sensitivity, as suggested by studies in transgenic mice with increased[45] or decreased[46] GR expression, human T-ALL cell lines with different GR levels[43] and GC-sensitive and -resistant multiple myeloma lines.[34] However, GR expression at the onset of the response may represent only part of this mechanism: Removal of GC within the first 24 h prevents cell death in ALL cells,[47] suggesting that sufficient GR levels need to be maintained for a considerable time. GR expression, however, is subject to negative feedback regulation, at least in cells not undergoing GC-induced apoptosis.[48] In contrast, in cells sensitive to the cytolytic effect of GC, evidence for GR autoinduction has been provided: In multiple myeloma lines, GC sensitivity and resistance correlated with induction[49] and repression[50] of GR mRNA, respectively. Elegant experiments exploiting tetracycline-regulated GR expression showed requirement of GR autoinduction for GC-induced apoptosis in CCRF-CEM derivatives,[51] and impaired GR autoinduction was observed in GC-resistant, but not in GC-sensitive, subclones of the same ALL model.[52] Moreover, Jurkat T-ALL cells, which, like GC-sensitive CCRF-CEM T-ALL cells, carry one wild-type and one mutated GR allele, are GC resistant and fail to autoinduce their GR.[53] Maintaining high GR levels through constitutive expression of transgenic GR leads to GC sensitivity in these cells.[44] Thus, at least in leukemia cell lines, maintenance of sufficient GR levels throughout a critical phase of the response appears mandatory (although not necessarily sufficient) for cell death induction, and this might be accomplished by GR autoinduction.

Gene transactivation, transrepression or both?

Although it is widely accepted that cell death induction by GC results from alterations in gene expression, it is still controversial whether it requires gene transactivation, transrepression or both. Mice carrying a dimerization deficient GR (GR^{dim} 'knockin' mice) are deficient in GC-induced thymocyte apoptosis,[41] suggesting that transactivation is required (although not necessarily sufficient in itself). A similar conclusion was derived from studies in GR-deficient S49 mouse thymoma cells transfected with N-terminal-deleted GR constructs.[54] In contrast, GC sensitivity could be restored in GC-resistant Jurkat[44] and CEM[55] human T-ALL cells by constitutive expression of transactivation-deficient GR mutants, suggesting that transrepression alone was sufficient for cell death induction (although a potential requirement for transactivation-dependent GR autoinduction would not have been detected in these studies because the GR was expressed from a strong constitutive promoter). More recently, we generated several transgenic subclones of the GR-deficient CEM-C7R1 cell line[42] with different expression levels of either GR^{wt} or GR^{dim} and found that the mutant conferred GC sensitivity only if expressed at considerably higher levels than the wild type (S Riml, in preparation). At these levels, the remaining transactivation potential of the mutant GR might suffice to induce critical target genes, a hypothesis currently being tested by comparative expression profiling. Thus, neither transactivation nor transrepression has been conclusively ruled out by the above studies, and it is possible that both mechanisms contribute to GC-induced cell death.

GC-regulated genes responsible for cell death induction

The key question of which GC-regulated genes are responsible for triggering cell death has been addressed by a number of classical gene search approaches (reviewed in Geley *et al*.[27]) without providing a generally accepted answer. Recent microarray-based expression profiling of cells undergoing GC-induced cell death has considerably increased the number of potential candidate genes.[29–38] An ongoing detailed bioinformatic meta-analysis of these publications (Schmidt *et al*., in preparation) revealed that not a single gene was found to be regulated in all eight investigated biological systems (cutoff: more than two-fold), and only a few appeared in three or more systems and/or publications (Table 1). Altogether, some 900 different genes were reported as GC regulated, but of these only $\sim$70 appeared in more than one publication. Although this small number might result from the use of arrays with only partially overlapping gene composition, technical or bioinformatic problems, and the way regulated genes were reported (in some papers only a selection of all regulated genes), it still suggests that a distinct set of genes might be regulated in different cell systems and experimental conditions. This raises the possibility that multiple, cell-context-dependent mechanisms rather than a conserved canonical pathway may lead to GC-induced cell death. However, since only about one-third of the human genome has been studied thus far, important genes may have been missed and a shared pathway may eventually be revealed.

Given the limitations noted above, the gene list in Table 1 is far from complete and thus cannot exclude additional

Table 1 Genes regulated by GC in cells prone to GC-induced apoptosis[a]

Identifier[b]	Description[c]	Reg[d]	Human[e]	Mouse[e]	Systems[f]
Hs.81328	NFkB inhibitor α (IκB-α)	↑	31,34,30,29,36[g],37	33, 32[h]	PreB, S49, WEHI, MM, Jurkat, CEM, thymus
Hs.7557	FK506-binding protein 5 (FKBP 51)	↑	31,34,35,30,37	33	PreB, WEHI, MM, EoL, Jurkat, CEM
Hs.84063	BCL2-like 11 (apoptosis facilitator) – Bim	↑	31,29,36[g]	33, 32[h]	PreB, S49, WEHI, CEM, thymus
Hs.420569	GILZ	↑	31,34,29	38[i]	PreB, MM, CEM, thymus-2
Hs.111244	HIF-1 responsive RTP801 (dig-2)	↑	36[g]	33, 32[h]	S49, WEHI, thymus, CEM
Hs.146393	Ubiquitin-like domain member 1	↑&↓	31	33, 32[h]	PreB, S49, WEHI, thymus
Hs.126608	Glucocorticoid receptor α	↑	29,36[g],37	33	PreB, S49, CEM
Hs.362807	Interleukin 7 receptor	↑	30,29,36[g]	38[i]	Jurkat, CEM, thymus-2
Hs.50640	Suppressor of cytokine signaling 1 (SOCS1)	↑	35,29,36[g],37		PreB, EoL, CEM
Hs.179526	Thioredoxin interacting protein (TXNIP)	↑	31,34,29,36[g]		PreB, MM, CEM
Hs.422550	Absent in melanoma 1	↑	31,30,29,36[g]		PreB, Jurkat, CEM
Hs.90708	Granzyme A	↑&↓	31,37	33	PreB, S49, WEHI
Hs.13291	Cyclin G2	↑&↓	31,29	33	PreB, S49, CEM
Hs.442669	Glutamine synthase	↑	31,34	38[i]	PreB, MM, thymus-2
Hs.75231	Solute carrier family 16, member 1 (MCT-1)	↓	31,30		Pre B, Jurkat, CEM
Hs.6241	PIP-3-kinase, regulatory subunit (p85 α)	↑	35	33	S49, WEHI, EoL
Hs.512712	Tubulin β polypeptide	↓	30	33	WEHI, Jurkat, CEM
M99054	Acid phosphatase type 5	↑		33, 32[h]	S49,WEHI, thymocytes
Hs.131924	G protein-coupled receptor 65	↑		33, 32[h]	S49, WEHI, thymocytes
D50683	TGF-β II Receptor α	↑	34,30		MM.1S, Jurkat, CEM
Hs.315562	Glutamate–cysteine ligase, modifier subunit	↑&↓	30,31		Pre B, Jurkat LS7, CEM
Hs.435051	CDK inhibitor 2D (p19, inhibits CDK4)	↑&↓	30,37		Jurkat, CEM, preB
Hs.118183	Hypothetical protein FLJ22833	↓		33, 32[h]	WEHI, S49, thymus
Hs.282326	Down syndrome critical region gene 1	↑	31,29,36[g],37		PreB, CEM
Hs.73958	Recombination-activating gene 1 (RAG 1)	↑&↓	31,29,36[g],37		PreB, CEM
Hs.202453	c-myc	↓	30,29,36[g]		Jurkat, CEM
Hs.443057	CD53 antigen	↑	31,29,36[g]		PreB, CEM
Hs.75462	BTG family, member 2	↑	31,36[g],37		PreB, CEM,
Hs.42322	Paralemmin 2	↑	31,36[g],37		PreB, CEM
Hs.528404	Integrin α 4 (antigen CD49D)	↓	31,29,36[g]		PreB, CEM
L19314	Human HRY gene, complete cds	↓	30,29,36[g]		Jurkat, CEM

[a]Genes are listed according to the number of systems wherein regulation was observed [b]Unigene number (starting with Hs.) or GeneBank accession number (all others) [c]Commonly used gene name [d]↑ and ↓ denote two-fold or greater gene induction or repression, respectively [e]References to human or mouse work, respectively [f]Cellular systems: *Human*: CEM, various subclones of the CCRF-CEM T-ALL cell line as specified in the respective publications; PreB, PreB-697 B-ALL cells; MM; multiple myeloma cell line MM1s; Jurkat; T-ALL cell lines stably transfected with either rat GR^{wt} or rat GR^{LS7} *Mouse*: WEHI, WEHI7.2 lymphoma cell line; S49, S49.A2 lymphoma cell line; thymus-1, normal C56BL/6 thymocytes; thymus-2, 18d fetal thymocytes from C57BL/6 wild-type mice or GR2KO mice [g]Reference Webb *et al*.[36] contains only genes regulated in CEM cells sensitive to GC-induced apoptosis but not those regulated both in GC-sensitive and GC-resistant cells. Thus, such genes may be particularly relevant for cell death induction [h]Although 59 genes were regulated, only seven were reported that were found to be regulated in S49 and WEHI as well[32] [i]Although many more genes were regulated, only 20 genes were reported in Mittelstadt and Ashwell[38], that is, those most strongly regulated in both mouse strains. Since GR2KO mice are resistant to GC-induced apoptosis, regulation of these genes may not suffice for cell death induction

possibilities or hypotheses. The list might, however, constitute the most informative collection of genes to date with strong evidence for regulation by GC in cells prone to GC-induced apoptosis. Regarding their function, these genes might be tentatively grouped into three classes: (i) genes directly implicated in death and survival decisions; (ii) genes whose (de)regulation might lead to cellular distress (thereby entailing apoptotic or (apo)necrotic cell death, as discussed in Figure 2 and (iii) genes not causal in the death response. The latter comprise three functionally distinct subgroups: genes that may counteract the apoptotic response (e.g., receptors for TGFβ or IL-7), others that may control clinically relevant GC effects such as cell cycle progression, and 'innocent bystanders'. Finally, regulation of the GR itself deserves separate mention, since its regulation determines extent and duration of all other regulatory responses. In the following, we discuss some of the evolving death pathways and current models. These pathways are not mutually exclusive; depending on the cellular context and other circumstances, either may be used preferentially or several may act in parallel in a single cell (Figure 3).

GC-induced apoptosis as result of direct regulation of death or survival genes

GC might directly activate the apoptotic machinery by regulating components of either the 'extrinsic' or 'intrinsic' pathways or both. Studies using the caspase 8 inhibitor *crmA* in transgenic mice[56] and human ALL cell lines[31,57] suggested that GC-induced apoptosis may not critically depend on the extrinsic pathway. However, in mouse thymocytes, FasL is induced by GC,[1,58] and Caspase-8 inhibition countered cytochrome *c* release and apoptosis.[59] Thus, depending on experimental circumstances, the extrinsic pathway may or may not contribute to GC-mediated death signaling. Evidence for involvement of the intrinsic pathway, particularly of members of the Bcl-2 family, has been provided in essentially all systems: GC apoptosis in thymocytes from APAF-1-[60,61] and caspase 9-[62,63] deficient mice is compromised (although not absent), and thymocytes from double knockout mice lacking the BH3-only molecules Bax and Bak[64] are GC resistant. Moreover, the single 'knockouts' of the BH3-only proteins Bim,[65] and Puma or Noxa[66] show partial GC resistance (Bax,[67] and Bid[68] knockouts cause mild, if any,

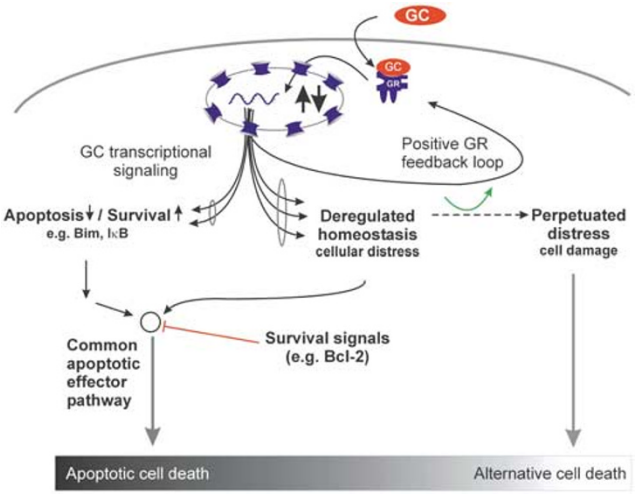

Figure 3 Proposed model for GC-induced apoptosis. GC may induce apoptosis by directly regulating typical apoptosis or survival genes, such as Bim or IkB (left side of the figure), or by inducing cellular distress that triggers the apoptotic cascade. In the presence of overexpression of antiapoptotic genes (such as Bcl-2), this mechanism may be blocked. If, under these circumstances, the GC-induced cellular distress is perpetuated by GR autoinduction, it may lead to (apo)necrotic cell death (right side of figure) (for further details see text)

deficiency in this response). Furthermore, overexpression of antiapoptotic Bcl-2 family members attenuated GC-induced cell death both in mouse thymocytes[69] and human ALL[47,70] and myeloma[71] cell lines. Since induction of proapoptotic[33,72] and repression of antiapoptotic[34,73] Bcl-2 family proteins has been observed in GC-treated cells, transcriptional deregulation of the Bcl-2 rheostat may be an essential principle for GC-induced apoptosis in many systems. However, as discussed below and depicted in Figure 3, the situation may become more complex in the presence of overexpressed antiapoptotic Bcl-2 family members or other prosurvival genes.

In addition to the regulation of components of the apoptotic machinery proper, GC may induce cell death by interfering with critical survival pathways. Perhaps the most intensively studied system is multiple myeloma, where interference with survival signaling through activation of the related adhesion focal tyrosine kinase (RAFTK; also known as Pyk2), a member of the focal adhesion kinase (FAK) subfamily, has been implicated in GC-induced apoptosis.[74] In support, IL-6 protected such cells from GC-induced apoptosis, and this was associated with RAFTK/Pyk2 inactivation mediated by the protein-tyrosine phosphatase SHP2.[75] However, GC regulation of RAFTK/Pyk2 was not observed in expression profiling studies of multiple systems (Table 1), suggesting that this mechanism might be specific for myeloma cells. Other examples for possible GC effects on survival pathways supported by the studies summarized in Table 1 include the induction of the NFkB inhibitor IkB[76] and of GILZ, which interacts with, and inactivates, NFkB[77] and AP-1.[78] Related DNA-binding-independent mechanisms (hence not readily detected by mRNA expression profiling studies) include direct protein–protein interaction of the GR with components of NFkB, AP-1 and other transcription factors like p53 implicated in death/survival decisions (reviewed in Geley *et al*.[27] and Herrlich[79]). Alternatively, or in addition, the above GR protein–protein interactions might account for the anti-inflammatory GC effects.[80]

Cell death as result of GC-induced cellular distress

Alternatively, or in addition to the above mechanisms, GC might induce apoptosis indirectly by gene (de)regulations that entail distress and cellular damage. This category might include the regulation of genes affecting metabolic pathways,[13,29] general transcription and/or translation,[30] production of, or response to, oxygen radicals,[81,82] Ca^{2+} fluxes[81,83] or intracellular pH[84] and volume control.[85] Examples from Table 1 that support these conclusions include the induction of the thioredoxin inhibitor, TXNIP, or repression of the lactate transporter, MCT-1. The former might contribute to increased oxidative stress, the latter to metabolic alterations, pH changes and/or disturbed volume control. As discussed in the Introduction, the resulting cellular distress may consecutively activate the apoptotic machinery. If apoptosis is blocked, for example, by overexpression of antiapoptotic Bcl-2 proteins or activated survival pathways, the cellular distress may become incompatible with cell survival and, if maintained for a sufficient time, lead to necrotic cell death. In support of this mechanism, high levels of transgenic Bcl-2 and Bcl-XL even in combination with saturating amounts of the pan-caspase inhibitor z-VAD failed to restore viability in GC-treated CCRF-CEM T-ALL cells in continuous presence of the drug. The resulting cell death was, however, delayed and showed altered morphology, including reduced DNA fragmentation and increased membrane permeability for vital dyes, as shown by time-lapse video microscopy (C Ploner, in preparation). Thus, there may be a continuous transition between two cell death forms, one rapid and showing typical apoptotic features in cells with low levels of Bcl-2 (and/or other antiapoptotic molecules) and another retarded form with more necrotic characteristics in cells with high levels of such proteins (Figure 3). The latter, slow cell death form may be critically dependent upon GR autoinduction (or at least lack of GR downregulation).

Mechanisms of resistance to GC-induced apoptosis

In general terms, GC resistance is defined as the inability of an individual cell or an entire organism to respond to all or a restricted number of GC responses. It can be absolute, as is the case in the absence of the GR, or relative and dependent on specific circumstances such as GC concentration, presence of apoptosis-inhibiting or -facilitating factors, etc. In the context of this review, GC resistance refers to the failure of lymphoid lineage cells to undergo GC-induced cell death under specific experimental or clinical conditions. Whether it affects other responses as well, whether it is context dependent or absolute, and what the underlying molecular mechanisms are, have considerable clinical consequences. For instance, if caused by GR gene mutations, GC resistance is absolute and irreversible, rendering the continuation of GC treatment with all its long-term side effects questionable. If

caused by regulatory mechanisms, therapeutic reversal of GC resistance might become an option.

An almost endless number of possible molecular mechanisms for GC resistance can be envisaged along the signal transduction pathways triggered by GC (Figure 4), and some of these mechanisms have recently been reviewed elsewhere.[5,8,9,52,86]) Conceptually, they may be grouped into 'upstream' and 'downstream' mechanisms. The former concern the GR, its ligand and GR-associated proteins that control its function, and have the potential to affect most, if not all, GC effects while the latter interfere with, and affect, only individual GC responses. In lymphoid malignancies, clinically relevant GC resistance means continuous expansion of tumor cells in the presence of GC, requiring resistance to both apoptosis induction and GC-mediated cell cycle arrest, processes that, at least in ALL cell lines, follow distinct pathways.[6] However, to simultaneously interfere with multiple pathways via 'downstream mechanisms' is considerably more complex than through 'upstream mechanisms'. Indeed, convincing evidence for a causative role in resistance to GC-induced apoptosis has so far mainly been provided for 'upstream mechanisms'.

'Upstream mechanisms-1': insufficient ligand

Most apical in the response is the requirement for sufficient intracellular levels of biologically active GC. This parameter is technically difficult to assess, but GC-like bioactivity can be determined in the plasma of patients during therapy.[87] Insufficient plasma levels may result from impaired uptake, increased steroid-binding proteins in the circulation or reduced converting enzyme activity, if prodrugs like prednisone are used. Intracellular GC levels may be reduced by overexpression of members of the large ABC transporter family, most notably the *mdr-1* gene- encoded P-glycoprotein

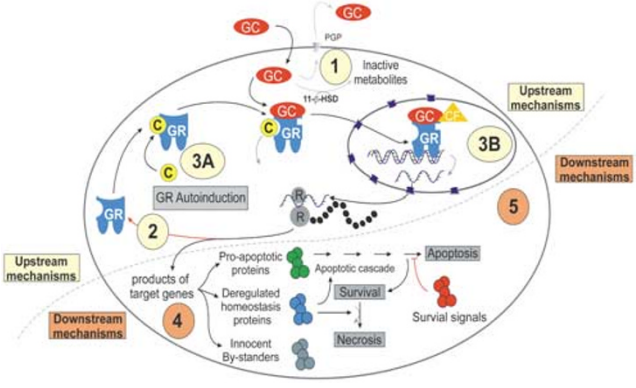

Figure 4 Principal mechanisms of GC resistance. Possible resistance mechanisms were organized along the GC signaling pathway and numbered consecutively: 'Upstream' mechanisms 1 and 2 have been detailed in the text, upstream mechanism 3 concerns deficiencies in GR-associated proteins in the cytoplasm (3A) and nucleus (3B), respectively (discussed in Kofler *et al*.[52]). 'Downstream' mechanisms encompass (4) defects in components of the specific response pathway or (5) crosstalk from other signaling pathways that interfere with and antagonize the death response. Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenase type 2; C, chaperones; CF, transcription cofactors, GC, glucocorticoid; GR, glucocorticoid receptor; Pgp, P-glycoprotein; R, ribosome

and the multidrug resistance-associated protein, MRP, as well as the lung-resistance protein (LRP), a major vault protein that formally does not belong to the ABC family but is still implicated in drug resistance (reviewed in Gottesman *et al*.[88]). In addition to affecting apoptotic responses to other agents as well, this form of GC resistance is characterized by its sensitivity to Pgp inhibitors, like verapramil or cyclosporin A, and its differing efficiency towards various GC analogues,[89] which might open therapeutic possibilities. *Mdr-1* gene overexpression has been made responsible for GC resistance in a mouse thymoma line,[90] but what role this form of GC resistance might play in patients is not clear.[8,88,91] Finally, GC resistance might be caused by expression of GC-metabolizing enzymes such as 11β-hydroxysteroid dehydrogenase type 2 that converts cortisol into inactive cortisone, as has been shown in rat osteosarcoma cells,[92] or mouse osteoblasts/osteocytes[14] transgenic for this enzyme.

'Upstream mechanisms-2': GR mutations, splice variants or insufficient expression

The next checkpoint in the pathway is the GR itself where mutations, occurrence of GR variants and insufficient expression might cause resistance. Numerous loss-of-function mutations in the GR gene have been observed in GC-resistant human ALL cell lines (e.g., Hala *et al*.[42] and Strasser-Wozak *et al*.[93]), but whether GR mutations constitute a major resistance mechanism *in vivo* remains unresolved. The combination of GC and chemotherapy, with its mutagenic potential, might indeed favor the development of, and subsequent selection for, GR mutations. However, one study found no evidence of mutations in the DNA- and ligand-binding domains of the GR in 22 chronic lymphatic leukemia patients subjected to combination chemotherapy,[94] and another study with $\sim$50 children with relapsed ALL provided only limited evidence for GR mutations as the cause of GC resistance (J Irving *et al*., submitted for publication).

GC resistance may also be caused by increased expression of GR variants (Figure 1) resulting from alternative splicing, polyadenylation or translational initiation, namely GR-β, GRγ, GR-P/GR-δ, GR-A and GR-B (for citations to the original literature see Tissing *et al*.[8] and Kofler *et al*.[52]). GR-P/GR-δ and GR-A were detected in a GC-resistant myeloma cell line, and GR-P in a number of hematopoietic and other malignancies as well as in normal lymphocytes, but how these variants might affect GC sensitivity remains controversial.[95,96] The GR-β splice variant reportedly encoded a dominant negative GR protein[97,98] and has been implicated in various forms of GC resistance, including patients with lymphoblastic malignancies.[99,100] However, there is little, if any, GRβ expression in various hematopoietic tumors, which makes its role in resistance development questionable.[50,96,101] Indeed, Haarman *et al*.[102] concluded that GRβ is not involved in GC resistance in childhood leukemia, although a possible involvement of GRγ in certain childhood leukemia subgroups could not be excluded. Whether GR-B affects sensitivity to GC-induced apoptosis in lymphoid malignancies is unknown.

GRα is the major functional GR isoform and, as discussed above, its expression is a critical factor for GC sensitivity in

numerous experimental systems. In clinical studies, GR expression levels above ~10 000 copies per cell at diagnosis correlated with beneficial outcome in childhood ALL,[103,104] but this correlation is not a consistent finding.[105,106] As discussed previously, GR levels at the onset of treatment may not be as important as GR expression kinetics (up- or downregulation) during treatment, but clinical studies addressing this question have not been reported thus far.

'Downstream mechanisms' interfering with death or activating survival signals

Theoretically, resistance to GC-induced apoptosis might result from unresponsiveness of, or mutations in, GC-regulated genes critical for death induction or from activation of genes and/or pathways interfering with the GC-induced death pathway (Figure 4). There are many reports in the literature on GC resistance by downstream mechanisms in experimental systems; however, in most, if not all, cases, the observed phenotype might be better referred to as reduced sensitivity rather than true long-term resistance with maintained clonogenic survival in the continuous presence of the drug. In patients, glutathione and glutathione *S*-transferase expression (reviewed in Tissing *et al*.[8] and Haarman *et al*.[86]) and alterations in the 'Bcl-2 rheostat' have attracted considerable attention. Regarding the latter, expression of Bcl-2 family members was investigated in numerous studies with somewhat conflicting outcomes. For instance, some investigators suggested that Bcl-XL[73] or the Bax-α:Bcl-2 ratio[107] might play a role in the protection of leukemic cells from GC-induced apoptosis, but one report found no alterations in Bax and Bcl-2 expression during *in vivo* chemotherapy,[108] and another concluded that neither Bcl-2 nor Bcl-XL, Mcl-1, Bax, Bad or Bak had prognostic significance in such children at diagnosis.[109] Interestingly, Bcl-2 was increased in relapsed ALL samples,[110] and downregulation of Bcl-2 or Bcl-XL by antisense oligonucleotides lead to sensitization of leukemia or myeloma cell lines, and freshly isolated myeloma cells from patients.[111,112]

Clinical significance and future perspectives

GC-induced apoptosis: therapeutic principle or surrogate marker?

In vitro and *in vivo* GC sensitivity are major prognostic factors in childhood ALL (reviewed in Tissing *et al*.[8] and Haarman *et al*.[86]). Children who respond well to an initial 8d monotherapy with prednisone in the BMF protocol have an excellent prognosis, whereas those who do not generally have an unfavorable outcome.[113] This correlation holds true for subgroups with poor outcome as well (infant ALL,[114] T-ALL,[115] Philadelphia chromosome-positive ALL[116]), that is, children with good prednisone *in vivo* response fare better than those with poor responses. In spite of this suggestive evidence, the crucial clinical question remains as to whether the cytolytic (and cytostatic) GC effect is, indeed, of additional therapeutic value or, alternatively, whether GC sensitivity simply defines a clinical entity that is particularly sensitive to conventional combination chemotherapy. In the former case, it is important to identify causes for GC resistance and develop improved therapy protocols that prevent and/or circumvent it. In the latter, GC should only be used for prognostic purposes but, because of its long-term side effects, might be withdrawn from therapy protocols.

Ethical reasons preclude clinical studies comparing protocols with and without GC to conclusively resolve this question. However, the deferral of GC from the initial month of induction therapy to the second month resulted in decreased event-free survival, and different types of GC (dexamethasone *versus* prednisolone) in induction and maintenance also influenced event-free survival (reviewed in Gayon and Carrel[4]). This clinical evidence, and the fact that GC provide an additional tool in the chemotherapeutic array, strongly argues for a critical therapeutic role of these steroids. Compared to other antileukemic drugs, GC have almost no acute side effects, lack cancerogenic activity and induce apoptosis that is relatively cell specific and independent of p53,[117,118] which is frequently mutated in hematopoietic malignancies. In spite of these advantages, interest in further investigating the therapeutic potential of GC is surprisingly low, perhaps because their antileukemic effect was discovered 50 years ago. Had this discovery been made in the era of Cleevec, interest in these compounds would probably be tremendous.

From a molecular understanding to the bedside: optimizing therapy protocols

Although patients with lymphoid malignancies may be treated successfully with existing protocols, there are many who are not, and even those who are cured suffer from considerable treatment-associated side effects, including the risk of secondary malignancies developing decades later, a threat particularly relevant in childhood ALL. There are numerous considerations centering around improving efficiency, reducing side effects and, most importantly, preventing or reverting GC resistance that may be present at the onset of treatment or develop during therapy (primary and secondary resistance, respectively). Current therapy protocols based on trial and error of a limited number of substances and combinations thereof are unlikely to represent optimal therapeutic regimens. Many compounds have been identified that potentiate the antileukemic GC effects, including histone deacetylase inhibitors,[119] immunophilin-targeting drugs,[120] immunomodulatory derivatives of thalidomide (IMiDs),[121] proteasome inhibitors such as PS-341,[122] and the anti-CD20 antibody rituximab.[123] These substances as well as new GC analogues with distinct pharmakokinetic properties (blood/brain or blood/testis barrier penetration; sensitivity to P-glycoprotein; etc.) might be combined in wide variety of ways with existing or emerging chemotherapeutics and drugs that target specific oncogenic pathways (for a review, see Anderson[124]). This complexity is further potentiated by an increasing number of entities among lymphoid malignancies, as defined by their expression profiles and polymorphic patient drug responses. Optimal protocols will need to be tailored to specific tumor subgroups and individual patients

('individualized medicine'). A profound molecular understanding combined with improved preclinical test models will be required to distill the almost infinite number of conceivable protocols to a few that can be subjected to clinical studies.

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