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Review

Advances in the understanding of the mechanisms of glucocorticoid sensitivity and resistance in lymphoid malignancies

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ABSTRACT

Glucocorticoids (GCs) represent an important component of treatment regimens for lymphoid malignancies including acute lymphoblastic leukaemia (ALL), multiple myeloma (MM) and chronic lymphocytic leukaemia (CLL). However, the exact mechanisms underlying their cytotoxicity are not well understood. Nor is it understood why some patients respond to GC treatment while others do not. Understanding the molecular mechanisms underlying GC sensitivity and resistance is important as it could provide the basis for novel therapeutic strategies. It is noteworthy that, although the cytotoxic effects of glucocorticoids on cells of lymphocytic origin have been known for decades, it is only over the last 10-15 years that we have begun to understand the molecular mode of action of GCs in lymphoid malignancies. Research interest in the subject, and in particular the mechanisms underlying GC resistance, has been growing steadily over this period, and it is now clear that GC-induced killing requires transcriptional activity resulting in either the induction of death-inducing genes or the repression of survival genes. Whilst GC-induced transcriptional activity and associated de novo protein synthesis are critical events in determining the cellular response to GCs, emerging evidence suggests that post-translational modification of certain proteins can also influence GC sensitivity. Conversely, defects in the GC-initiated apoptotic signalling pathway can contribute to glucocorticoid resistance. In this review, we will discuss the molecular mechanisms underlying cellular responsiveness/resistance to glucocorticoid-induced cytotoxicity in lymphoid malignancies and highlight the similarities and differences between GC sensitivity/resistance in CLL, ALL and MM.

KEYWORDS: glucocorticoids, cytotoxicity, apoptosis, B-lymphocytes, leukaemias

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1. Glucocorticoid therapy in lymphoid malignancies: Historical perspectives

1.1. Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia (ALL) is the most common malignancy in children. Despite the fact that more than 80% of paediatric ALL patients are cured nowadays thanks to the remarkable improvement in the outcome of therapy since the 80s, it remains the leading cause of cancer-related death in children [1, 2]. ALL is characterised by accumulation of immature lymphoid precursor cells in the bone marrow, peripheral blood, and central nervous system with B-lymphoblastic (B-ALL) contributing to 85% of all cases. Genetic abnormalities including aneuploidy and chromosomal rearrangements are common and can be detected in more than 75% of cases [3-5]. It is noteworthy that detection of these various genetic abnormalities and molecular alterations plays an important role in identifying and managing high-risk subgroups of ALL patients. For instance, the t(9;22) chromosomal translocation (also known as Philadelphia chromosome) which gives rise to an oncogenic BCR-ABL1 fusion protein is associated with poor prognosis. Although it is not a common genetic defect in childhood ALL (2-4%), almost 25-30% of adult patients with ALL carry the Philadelphia chromosome and consequently express the constitutively active BCR-ABL1 tyrosine kinase [4, 6, 7]. New kinase inhibitors such as imatinib and dasatinib specifically targeting tyrosine kinases including BCR-ABL1 have now been incorporated into treatment regimens and have shown promising clinical activity [7, 8].

Glucocorticoids such as prednisolone, hydrocortisone and more recently dexamethasone have remained key components in the treatment of ALL for over half a century ever since they were shown to be able to induce remission in childhood ALL in the 1950s [9, 10]. Indeed, the initial response to GCs has been considered as a major prognostic factor as resistance to GCs was shown to be associated with relapsed/refractory ALL [11-13].

1.2. Multiple myeloma

Multiple myeloma (MM) is a malignant disorder characterized by the monoclonal expansion of plasma cells derived from B lymphocytes in the bone marrow and secretion of monoclonal immunoglobulin [14, 15]. Clinical features of this disorder include hypercalcemia, renal insufficiency, anaemia, and bone lesions (also known as CRAB) [16].

For many years, vincristine, doxorubicin and dexamethasone were used as a primary induction therapy for newly diagnosed patients with MM [17]. However, over the last decade significant advances have been made in the treatment of MM owing to the introduction of innovative regimens that incorporate the proteasome inhibitor bortezomib and the immunomodulatory drugs thalidomide and lenalidomide [14, 18]. Nonetheless, GCs remain an integral part of the modern therapeutic regimens. Despite the improvement in the clinical outcome with the newer therapies, MM is still incurable as not all patients respond to treatment and even those who respond initially will ultimately relapse [18-20], and effective treatment of relapsed/ refractory MM still remains a challenge.

1.3. Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) results from a clonal expansion of antigen-experienced B cells with a distinctive immunophenotype that accumulate in the blood, bone marrow, liver and spleen [21, 22]. It is the most common leukaemia in adults in the Western countries and well recognised for its clinical variability between individual patients [22]. Many CLL patients can have an indolent course without the need of treatment for many years, whereas others progress very rapidly and succumb to the disease within a few years from diagnosis. This variability also extends to therapeutic response in that some

patients with CLL respond well to frontline fludarabine-based chemotherapy, while many others fail to respond to the conventional therapy and require alternative treatment.

Unlike the situation in ALL and MM, GCs have had chequered history in CLL as clinical trials in the 70s and 80s showed no benefit of adding conventional-dose prednisolone to chemotherapy [23, 24]. Consequently, GC treatment was confined to patients with autoimmune complications [25]. However, GCs were re-introduced into the clinic as definitive therapy in the 90s following *in-vitro* [26] and *in-vivo* [27, 28] studies showing that high-dose methylprednisolone (HDMP) is active in relapsed/refractory CLL. Promising results have been obtained by combining HDMP or dexamethasone with rituximab in the refractory [29-32] and frontline [33] setting. Encouraging results have also been obtained with HDMP in combination with alemtuzumab in CLL patients with *TP53* defects [34, 35]. Therefore, glucocorticoids (GCs), either alone or in combination with other agents, have emerged as a useful and important treatment option for patients with chemoresistant or *TP53*-defective CLL, which is in keeping with their p53-independent mechanism of action [36, 37].

2. Mechanisms of glucocorticoid action

Therapeutic GCs such as prednisolone, 6-methylprednisolone, hydrocortisone and dexamethasone are analogues of cortisol, a steroid hormone secreted by the adrenal cortex in response to stimulation by the pituitary adrenocorticotrophic hormone [38]. Cortisol plays a key physiological role in limiting the inflammatory response and regulating immune function, and therapeutic GCs mimic this activity [39].

2.1. Glucocorticoid receptor signalling

GCs exert their effects by binding to the cytoplasmic GC receptor (GR) (Figure 1), a member of the nuclear hormone receptor family. The GR comprises three major functional domains, an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) [40, 41]. In the absence of GCs, the cytoplasmic GR forms a heterocomplex with chaperone proteins such as heat shock protein (hsp) 90 and 70, which keeps the GR in an inactive state. Binding of GR by GCs dissociates GR from its molecular chaperones, allowing its formation of a homodimer via the C-terminal LBD and subsequent translocation into the nucleus [42].

Once in the nucleus, the GR homodimer interacts with specific DNA sequences known as GR response elements (GRE) in the promoter regions of its target genes [43]. The GRE-bound GR then recruits co-activator proteins that modify chromatin structure and assemble the necessary transcription machinery, resulting in the transcriptional activation or suppression of target genes [44, 45]. In addition to its direct effect on gene expression through DNA binding, the GC-GR complex can also regulate gene expression indirectly by interacting with other transcription factors, most notably NF-κB and AP-1 (Figure 1) [45, 46].

2.2. Glucocorticoids-induced apoptosis

Among the many important biological effects mediated by GCs is the potent and selective induction of lymphoid-cell apoptosis, which underpins their therapeutic application in lymphoid malignancies.

Two distinct apoptotic pathways have been identified - the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) death pathway [47, 48].

In the extrinsic pathway, apoptosis is initiated through ligand binding to cell surface receptors of the tumour necrosis factor (TNF) family such as TNF-R1 and Fas. Ligation of these receptors triggers formation of a death-inducing signalling complex (DISC), which consists of adaptor molecules such as the Fas-associated death domain protein (FADD) and procaspase-8. Within the complex, caspase-8 undergoes autoproteolytic activation. Active caspase-8 then activates downstream caspases such as caspase-3 and -7, leading to orderly degradation of intracellular substrates and cell death. The intrinsic pathway is activated when the integrity of the outer mitochondrial membrane is lost in response to diverse apoptotic stimuli. This results in the release of cytochrome *c* into the cytoplasm, where it binds to apoptotic protease-activating factor 1 (Apaf-1). Apaf-1 in turn recruits procaspase-9 to form a multimeric complex where the autoproteolytic

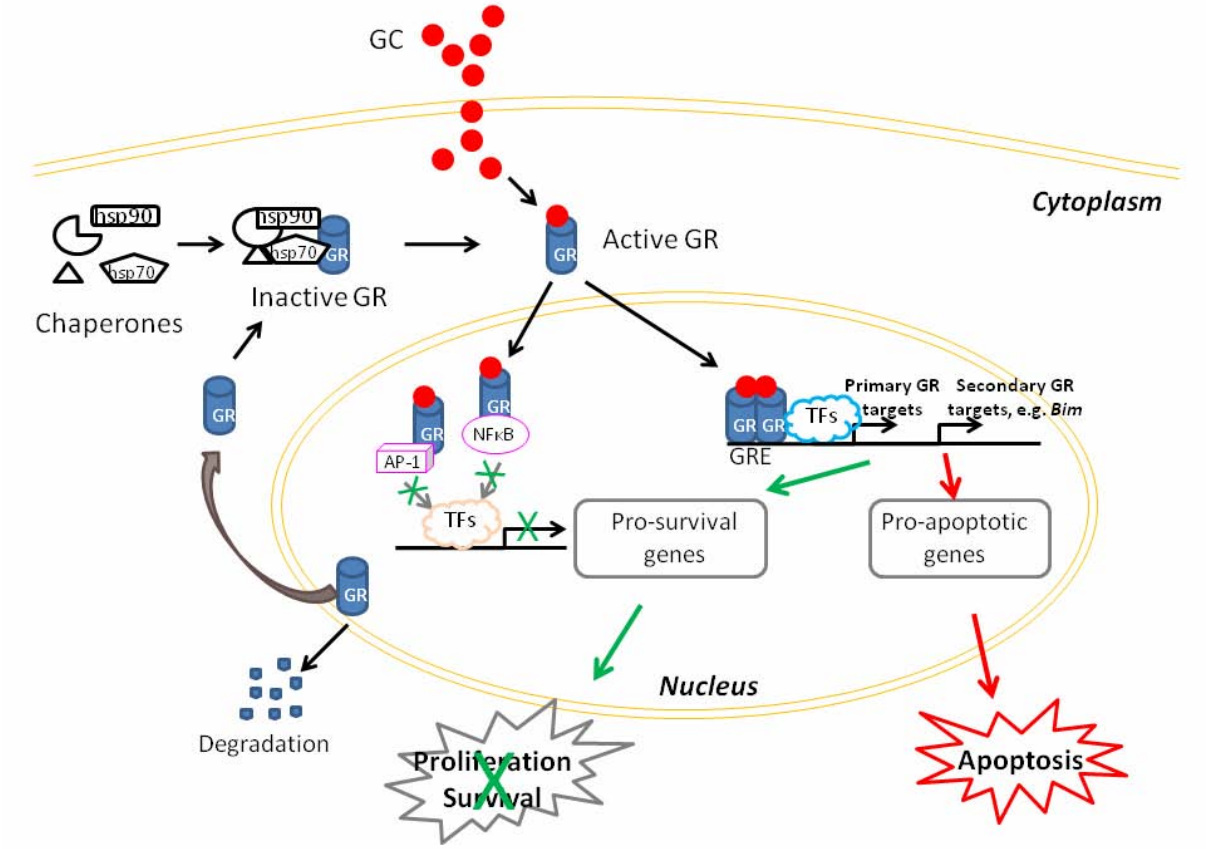

Figure 1. Mode of action of glucocorticoids in lymphoid cells. Glucocorticoid (GC) diffuses into cells and binds to the cytoplasmic GC receptor (GR). In the absence of GC, the GR is kept inactive by the chaperone proteins such as heat shock proteins (hsp) 90 and 70. Binding of GR by GC dissociates GR from the complexes with chaperone molecules, allowing the formation of a homodimer and its translocation into the nucleus. Once in the nucleus, the GR homodimer interacts with GR response elements (GREs) in the promoter regions of its target genes and acts in concert with other transcription factors (TFs) resulting in the activation (arrows in red) or suppression (arrows in green) of target genes in a cell type-specific manner. In addition, GC-GR complex can also regulate gene expression indirectly through interacting with transcription factors such as NF-κB and AP-1 (see text for more details).

activation of caspase-9 occurs. The active caspase-9 then efficiently activates other downstream caspases, bringing about the biochemical and morphological changes characteristic of apoptosis. This intrinsic pathway is mitochondria-dependent and tightly controlled by interactions between pro- and anti-apoptotic Bcl-2 family proteins [49]. The pro-apoptotic BH3 (Bcl-2 homology domain 3)- only proteins are considered as essential initiators of the mitochondrial death pathway [50].

The mechanisms underlying GC-induced apoptosis are not fully understood. Early studies using mouse thymocytes and primary cells and cell lines derived from patients with ALL and MM showed that GC-induced apoptosis is critically dependent on both the structural integrity and level of expression of the GR [51, 52]. It was subsequently shown that GC-induced apoptosis requires *de novo* gene expression and protein synthesis [53, 54]. Studies employing different types of lymphoid cells have shown that GCs alter the expression of a large number of genes. Although the identity of these GC-regulated genes varies widely between different studies [55], alteration in the expression of Bcl-2 family proteins has emerged as a common theme in lymphoid cells.

There is now overwhelming evidence suggesting that GCs induce apoptosis via the intrinsic pathway in lymphoid cells. First, GCs can induce expression of the BH3-only proteins Bim and Puma in malignant lymphoid cell lines [56, 57] as well as primary leukemic cells from patients with ALL [58] or CLL [59, 60]. Observations from animal studies have shown that lymphoid cells from mouse deficient of both Bim and Puma are resistant to GC-induced apoptosis [61, 62], suggesting that Bim and Puma are required for GC-induced apoptosis *in vivo*. Second, GC-induced apoptosis in leukemic cell lines and primary malignant cells from patient with ALL and MM is accompanied by reduced expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL [58, 63], suggesting a role for these proteins in protecting leukemic cells from GC-induced apoptosis. Thirdly, both splenic B cells and thymocytes from mouse deficient of both Bax and Bak are resistant to GC-induced apoptosis [64, 65]. Since Bax and Bak are required for the execution of the mitochondrial death pathway [66], the observations provided strong evidence that GCs-induced apoptosis is activated through this pathway. Finally, GC-induced apoptosis is significantly reduced in thymocytes from mice deficient in Apaf-1 [67] or caspase-9 [68, 69], both of which are involved in the execution phase of mitochondrial death pathway.

Despite this compelling evidence that GC-induced apoptosis involves the mitochondrial death pathway, there are a number of uncertainties regarding the exact mechanisms involved. First, no recognised GRE has hitherto been detected in the promoter of gene encoding for the BH3-only protein Bim [57, 70] or Puma [71]. Exact how GCs increase the expression of these genes therefore remains unclear. It is known that the GR can exert its biological effects in malignant lymphoid cells by regulating the expression of microRNAs (miRs) [72], and it has recently been shown in T-lymphoid leukaemia cells that GCs induce Bim expression by down-regulating the miR-17~92 microRNA cluster which is an endogenous repressor of Bim expression [73]. Whether this is a common mechanism by which GCs induce Bim expression remains to be seen.

Another possibility is that Bim or Puma is induced as a secondary response to the activation of a transcription factor that is directly transactivated by the GR. For example, a recent study showed that the c-Jun transcription factor, a known GR target gene, binds to promoter of *Bim* gene after GC treatment [74]. It has also been shown that Puma, together with Bim, can be induced in a p53-independent manner by the transcription factor FOXO3a [71], which is required for the GC-induced apoptosis of mouse splenocytes and in cell lines of lymphoid origin [75]. However, it is not clear how the GC/GR complex regulates FOXO3a expression. A further consideration is that, although the BH3-only proteins Bim and Puma are both required for GC-induced apoptosis in lymphoid cells *in vivo* [61, 62], their respective contribution to GC-induced apoptosis appears to be cell-lineage specific. For example, immature pre-B cells from the bone marrow of Bim knockout mice are completely protected from GC-induced apoptosis [61], suggesting an essential role for Bim and a redundant role for Puma in mediating GC-induced killing in these cells.

3. Mechanisms of glucocorticoid resistance

As described above, GCs exert their effects through binding to the GR and activating its transcriptional activity, resulting in downstream signalling events. Therefore, resistance to GC therapy can potentially occur through multiple mechanisms including reduced GR expression, loss-of-function mutations in the GR and defects in signalling pathways downstream of the GR. As resistance to GC-induced apoptosis limits the effectiveness of GC therapy, it is important to understand molecular mechanisms of GC resistance in order to develop novel strategies to overcome such resistance. It is beyond the scope of this review to include all published findings regarding general aspects of GC resistance as these have been extensively reviewed elsewhere [40,76-78]. Instead, it is our intention to focus on the discussion of recently published experimental and clinical evidence that could impact on our understanding of GC resistance in lymphoid malignancies specifically.

3.1. Reduced expression of glucocorticoid receptors

The GR is a ubiquitously expressed protein which is found in almost all mammalian cells including lymphocytes [54]. The sensitivity of malignant cells to GCs appears to correlate with the number of receptors found within the cell. Several groups have shown that reduced GR expression in primary ALL cells is associated with early resistance to GC therapy, relapse, and poor prognosis [11-13]. Furthermore, an increase in the number of GR molecules upon GC treatment, a phenomenon also known as GR auto-induction, has been considered as an important positive-feedback mechanism in GC-induced apoptosis. This mechanism is likely explained by the existence of GREs within the GR promoter regions [40]. Thus, work using T-cell lines showed that cells that exhibit GR auto-induction at the protein and/or mRNA level are sensitive to GC-induced apoptosis [79, 80], whereas cells that fail to induce the GR upon GC treatment are resistant [81].

However, these findings are in conflict with other studies using primary cells from ALL patients where reduced GR expression is rare and GC resistance is linked neither to an inability of resistant cells to up-regulate the expression of the GR upon GC exposure, nor to differences in *GR* promoter usage [82].

3.2. Mutations in the glucocorticoid receptors

Following the publication of clinical observations describing several endocrinological glucocorticoid resistance syndromes that are causally linked to genetic mutations in the GR [83, 84], efforts were made to identify similar mutations in lymphoid malignancies as a possible cause of GC resistance. A mutation in one GR allele (L753F) was found in the leukemic cells from a patient with ALL and clinical evidence of GC resistance [85]. This mutation introduces a premature stop codon, resulting in loss of GR expression. Other mutations in the GR gene have also been reported to correlate with decreased GC sensitivity [86]. However, in a separate study based on mutational screening of all coding exons of the GR gene in a cohort of 50 patients with relapsed ALL, the authors found that somatic mutations in GR are rare at relapse [87]. Another study also showed that somatic mutations were rare at diagnosis and remission [88]. These results therefore argue against the role of GR mutations as a major cause of GC resistance in ALL.

Several single nucleotide polymorphisms (SNPs) in the GR gene have been associated with altered GC sensitivity. The ER22/EK23 mutation within GR exon 2, which is associated with an elevated ratio of GR-α A to GR-α B, has been correlated to resistance to GC therapy [89]. As GR-α B is more transcriptionally active than GR-α A [90], the shift of GR-α A to GR-α B expression ratio may explain the observed effect on GC resistance. In contrast, the N363S SNP, which also occurs in exon 2 of GR gene, is associated with increased GC sensitivity [91]. The N to S substitution appears to alter interaction between GR and transcriptional coregulators [92]. However, none of these GR variants appear to be linked to GC sensitivity or resistance in childhood ALL [88].

3.3. Differential post-translational modification of glucocorticoid receptors

There is accumulating evidence that the activity of the GR is also affected by post-translational modifications (PTM) including phosphorylation, acetylation, nitrosylation, ubiquitination, and SUMOylation.

In essence, the GR is a phosphoprotein which can be phosphorylated at multiple sites by various kinases such as mitogen activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs), and differential phosphorylation is associated with differential GR function [78, 93]. For example, the transcriptional activity of the GR is increased when Serine 211 (S211) is phosphorylated [94]. Consequently, S211 phosphorylation is often regarded as a biomarker for GR activation [94]. In lymphoid cells, S211 of the human GR can be phosphorylated by p38 MAPK and this phosphorylation enhances GRE-dependent transcription [95]. In the same study, cells transfected with mutant GR (mutation of serine 211 to alanine to prevent phosphorylation at the position) displayed diminished apoptosis in response to GC treatment, demonstrating the functional importance of p38 MAPK-mediated

S211 phosphorylation in GC-mediated cell death. In contrast, phosphorylation of S226 by c-Jun N-terminal kinase (JNK) has been shown to inhibit GR transcriptional activity and also promote GR export from the nucleus [96, 97]. It is therefore unsurprising that inhibition of JNK can enhance GC-induced apoptosis in lymphoid cells [95]. Meanwhile, phosphorylation of S404 of the GR by glycogen synthase kinase-3β (GSK-3β) has been shown to facilitate its export into the cytoplasm, leading to enhanced GR degradation, and high level of S404 phosphorylation correlates with reduced GC-induced apoptosis in osteosarcoma U-2 OS cells [98]. It will be interesting to see whether high levels of GSK-3-mediated phospho-S404 in the GR are also present in some lymphoid cells and, if so, whether such cells are resistant to GC-induced apoptosis.

The GR can be acetylated at lysine (K) 494 and K495 in the DNA binding domain following dexamethasone (Dex) treatment, and acetylated GR failed to elicit Dex-induced repression of NF-κB-dependent gene expression in human lung adenocarcinoma A549 cells [99]. In the same study, histone deacetylase 2(HDAC2)-mediated GR deacetylation enabled the GR to bind the NF-κB complex and repress NF-κB-mediated gene expression. More significantly, overexpression of HDAC2 in GC-insensitive alveolar macrophages from patients with chronic obstructive pulmonary disease restored GC sensitivity in these cells. It remains to be seen whether GR acetylation is also associated with GC resistance in lymphoid cells.

Small ubiquitin-related modifier (SUMO) proteins (e.g. SUMO-1 consists of 98 amino acids with a MW of 11 KDa) are structurally related to ubiquitin and, in mammals, there exist four isoforms, namely SUMO-1, -2, -3 and -4 [100, 101]. Covalent binding of SUMO molecules to the GR has been identified at multiple sites in both the NTD and LBD domains with distinct functional consequences on protein stability, sub-cellular localisation and transcriptional activity [102, 103]. SUMOylation at lysines 277 and 293 in the NTD domain appears to negatively regulate GR transcriptional activity as substitution of lysine to arginine in these SUMO consensus motifs led to increased transcriptional activity [102]. In addition, overexpression of SUMO-1 can enhance GR degradation [103]. Recently, it has been shown that SUMO-dependent transcriptional inhibition depends on the integrity of the DBD domain and binding of the GR to DNA in a gene-specific manner [104]. It is intriguing that GR SUMOylation is also stimulated by JNK-mediated phosphorylation [105], suggesting that JNK may inhibit GR activity through multiple mechanisms including inducing many different PTMs of the GR, resulting in GC resistance. Therefore, although the precise mechanisms through which SUMOylation regulates GR transcriptional activity are not clear, inhibition of JNK may be a potential strategy to overcome GC resistance.

Another important PTM of the GR is the covalent addition of ubiquitin (Ub) to the receptor, thereby marking it for proteasomal degradation. This modification is believed to be critical in regulating GC signalling by controlling the rate of degradation of the GR [106]. Ubiquitin is a highly conserved small molecule (76 amino acids) that is ubiquitously expressed in all eukaryotic cells. It labels target proteins for proteasomal degradation through enzymatic covalent linkage, leading to recognition by the 26S proteasome [107]. The observation that proteasomal inhibition by chemical inhibitors stabilises the GR and enhances its transcriptional activity led to identification of the PEST motif (Proline [P], Glutamine [E], Serine [S] and Threonine [T]) in the GR at amino acids 399-419 with the lysine residue at position 419 (K419) as a ubiquitination site [108, 109]. The ubiquitinated PEST motif is a signal for proteolytic degradation by the 26S proteasome [110]. The requirement of K419 in the proteasoemal degradation of the GR is demonstrated by mutagenesis study where the K419A mutant GR displayed enhanced transcriptional activity and resistance to proteosomal degradation [109]. Furthermore, GR phosphorylation appears to be coupled to ubiquitination as mutation of serine residue within the PEST motif led to increased GR stability [106]. Understanding the effects of proteasome inhibition on GC sensitivity has clinical relevance as new therapies combining proteasome inhibitor

bortezomib and GCs such as dexamethasone, in addition to thalidomide, prove to be highly effective in the treatment of refractory/relapsed MM [111].

3.4. Defects in signalling pathways downstream of glucocorticoid receptors

As mentioned earlier, there clearly exists a discrepancy between clinical observations and those obtained using cell lines where GC resistance was often associated with defects at the level of receptor. This led to an intensive search for other causes of GC resistance distinct from those due to GR defects. Studies employing an experimental model of childhood B-ALL where primary leukaemia cells were transplanted into non-obese diabetic/severe combined immunodeficient mice revealed that GC resistance was caused by defects downstream of the GR but upstream of Bim induction [112, 113]. Specifically, GC-resistant cells displayed normal GR function when compared to sensitive cells, but failed to express Bim upon GC treatment. Exactly why the GC-resistant B-ALL cells were unable to up-regulate Bim expression in response to GC treatment is not clear. However, it has recently been shown that GC resistance in B-ALL xenografts models and patient samples correlated with reduced acetylation of histone H3 [114]. Conversely, chemical inhibition of histone deacetylase restored Bim expression and showed synergistic anti-leukemic efficacy with dexamethasone *in vitro* and *in vivo,* as described in the same study.

We and others have shown that Bim is up-regulated during the GC-induced apoptosis of CLL cells [59, 60] and we have further demonstrated that GC-induced killing of CLL cells depends on Bim upregulation [60]. Interestingly, GC treatment up-regulated Bim to comparable levels in both GC-resistant and sensitive cells but upregulated Bim was unable to activate Bax and Bak in resistant cells. This suggests that in CLL, GC resistance results from a blockade in the mitochondrial death pathway between up-regulation of Bim and Bax/Bak activation. The molecular mechanisms responsible for this blockade are not clear and currently under investigation in our laboratory.

4. Similarities and differences between GC sensitivity and resistance in lymphoid malignancies

In ALL, variation in GR expression and mutations of the GR, which are not common in the primary leukaemia cells, do not appear to correlate with GC sensitivity or resistance. Instead, GC-induced apoptosis appears to critically depend on the induction of the pro-apoptotic BH3-only protein Bim. Evidence to support this notion was first provided by experiments showing that knockdown of Bim by small interfering RNA (siRNA) or small hairpin RNA (shRNA) rendered the GC-sensitive cells resistant to GC-induced apoptosis [115, 116]. Further experimental and clinical studies demonstrated a strong correlation between GC resistance and failed up-regulation of Bim after GC treatment [112, 113, 117]. However, due to the lack of recognised GRE in the promoter region of the *Bim* gene, it remains unclear how Bim is upregulated by GR.

In contrast to ALL, in MM the integrity and expression level of the GR appear to be the main determinants of GC sensitivity [118]. Potential mechanisms responsible for altered GR expression and function include defective transcriptional activity of the GR [119] and overexpression of miR-130b [120]. miR-130b has been shown to bind to 3'-untranslated region of GR-α mRNA and its overexpression caused reduction in expression and decreased transcriptional activity of the GR, resulting in resistance to GC-induced apoptosis [120]. At the functional level, the ability of the GR to induce gene expression by binding to GREs seems a prerequisite for the induction of apoptosis by GC in myeloma cells [121]. However, the requirement of Bim in this process has not been proved conclusively. One study has shown that Bim expression is not sufficient to induce apoptosis since cells expressing a mutant GR incapable of eliciting GRE-dependent transactivation were resistant to GC-mediated apoptosis despite the preservation of Bim up-regulation [121]. In a separate study, gene silencing of Bim by siRNA significantly inhibited dexamethasone-induced apoptosis, indicating that Bim is a key mediator of GC-induced apoptosis in MM cells [122].

Therefore, the jury is still out as to whether Bim is essential for the GC-induced apoptosis of MM cells. However, it is likely that other molecules such as Puma may play a role, either on their own or in co-operation with Bim.

Similar to ALL and MM, the GC-induced killing of CLL cells occurs by apoptosis [123], requires caspases [124, 125] and involves conformational changes of Bax and Bak [126], consistent with activation of the mitochondrial death pathway. The potential role of GR alteration as a cause of GC resistance is inconclusive. One study did not detect any defects in the GR ligand-binding or DNA-binding domains in any of the 22 cases studied [127], suggesting that mechanisms other than altered ligand or DNA binding of the receptor are responsible for the lack of GC sensitivity. Another study of a single CLL patient with GC resistance found marked over-expression of the dominant negative GR-β splice variant but provided no experimental evidence linking the isoform to GC resistance [128]. Transcriptional activation of Bim has been implicated as a possible trigger of GC-induced apoptosis [59]. Our group has recently confirmed that Bim is required for the GC-induced apoptosis of CLL cells as knockdown of Bim by siRNAs reduced GC-induced upregulation of Bim protein and conferred resistance to GC-induced killing in previously GC sensitive cells [60]. Furthermore, we showed that, unlike ALL, GC resistance in CLL is not associated with reduced Bim expression as GC treatment resulted in the upregulation of Bim mRNA and protein to comparable levels in both GC-resistant and sensitive cells. Instead, GC resistance was associated with failed activation of Bax and Bak, indicating that GC resistance results from a blockade in GC-mediated apoptotic signalling between Bim up-regulation and Bax/Bak activation.

5. Potential strategies to overcome glucocorticoid resistance

The activity of GCs is influenced by a range of factors such as its accessibility to the GR, expression of the GR, sub-cellular localisation of the GC-GR complex and transcriptional function of the GR (Figure 1). In addition, GC signalling is a complex process that involves crosstalk with many other signalling pathways and takes place in a cell type-specific manner [78, 129]. It is therefore likely that GC resistance could arise from multiple mechanisms occurring at each step along the signalling pathways initiated by the GC-GR complex rather than from a single cause. Accordingly, attempts to devise a strategy to overcome such resistance will have to take into account the multitude of effects by manipulating the GC signalling for therapeutic gain. Consequently, two rational approaches can be contemplated in tackling the phenomenon of GC resistance in lymphoid malignancies. One is to restore and/or stabilise the expression of the pro-apoptotic molecules that are usually activated in response to GC, and the other is to target the pro-survival molecules that are regulated by GC signalling pathway.

5.1. Restore the expression of and stabilise pro-apoptotic molecules

As described earlier, induction of Bim expression by GCs appears to be a critical event in the GC-induced killing of some types of malignant lymphoid cells and failed induction of Bim is associated with GC resistance. The expression of the pro-apoptotic BH3-only protein Bim can be regulated at transcriptional, translational and post-translational levels [50, 130]. Bim is known to be phosphorylayted at many sites by different kinases resulting in distinct biological consequences [131]. MAPK/ERK (extracellular signal-regulated kinase) signalling pathways are often constitutively activated due to mutational activations of oncogenes or chromosomal translocations and deliver pro-survival signals to leukemic cells, and their activation has been linked to resistance to GC-induced apoptosis in malignant lymphoid cells [132, 133]. One mechanism of MAPK/ERK-mediated resistance to GC involves the direct phosphorylation of Bim by ERK, resulting in proteasomal degradation of Bim [134, 135] and inactivation of Bax [136]. Consequently, targeting MAPK/ERK pathways could be an important approach in suppressing pro-survival networks that often renders leukaemia therapy ineffective [137]. Indeed, pharmacological inhibition of MAPK/ERK restored the expression of Bim and

sensitised leukemic cells to GC-induced apoptosis in ALL and MM cells [138, 139]. In addition, apoptosis induced by histone deacetylase inhibitors (HDACi) in CLL cells is associated with up-regulation of Bim and Noxa [140, 141], although it is not clear whether HDACi can sensitise leukemic cells to GC-induced apoptosis. Noxa is a BH3-only protein which selectively interacts with and antagonises the function of Mcl-1, and intensive efforts are being made to develop small molecules mimicking the action of Noxa as a novel therapy in leukaemia [142, 143].

5.2. Target pro-survival Bcl-2 family proteins

Many pro-survival Bcl-2 family proteins such as Bcl-2 and Mcl-1 are overly expressed in different types of malignant lymphoid cells. In particular, higher levels of Bcl-2 and Mcl-1 have been associated with resistance to GC therapy in ALL [144-147]. In MM, the survival of leukemic cells was dependent on the expression of Mcl-1 [148, 149] and its overexpression was linked to relapse and short survival [150]. Also, it is well recognised that CLL cells express elevated levels of Bcl-2 and Mcl-1 proteins and that overexpression of each of these proteins is associated with poor response to chemotherapy and short survival [151, 152]. Therefore, small molecules targeting anti-apoptotic Bcl-2 family proteins could provide a novel approach to overcome GC resistance, although early indications suggest that they are highly active as therapeutic agents in their own right.

A small molecule inhibitor, ABT-737, which mimics action of the BH3-only proteins in interacting with and inhibiting the activity of Bcl-2 and Bcl-xL [153], has shown promising preclinical activity in ALL [154, 155]. Of particular interest, ABT-737 appears to have antileukemic activity in a mouse xenografts model of chemoresistant, GC-resistant ALL [154]. ABT-737 also demonstrated promising preclinical activity in MM [156-158] despite the established dogma that MM cells depend on Mcl-1 for their survival. Mechanistic studies revealed that a significant proportion of MM cells also relied on Bcl-2/Bcl-xL for their survival and ABT-737 is particularly effective in such MM cells [159, 160]. ABT-737 has also been shown to kill CLL cells effectively *in vitro* [153, 161, 162] and act synergistically with dexamethasone [163]. A phase I trial of ABT-263 (also known as navitoclax which is closely related to ABT-737 and orally bioavailable) in patients with refractory/relapsed CLL also demonstrated promising anti-leukemic activity as a single-agent [164].

Another small molecule pan-Bcl-2 family inhibitor, obatoclax (GX15-070), was developed as it has been shown to block BH3-mediated binding of Bcl-2, Bcl-XL, Mcl-1 and A1 to Bax and Bak, and in particular, antagonise Mcl-1 and overcome Mcl-1-mediated resistance to apoptosis [165, 166]. Significantly, obatoclax can restore GC sensitivity in previously GC-resistant ALL cells through the induction of both autophagy and apoptosis [167, 168]. In a preclinical study using a panel of myeloma cell lines, obatoclax was found to induce apoptosis through upregulation of Bim and synergise with dexamethasone in inducing cell death in many of the cell lines tested [157]. Also, a single-agent phase I trial of obatoclax has been conducted in heavily pre-treated patients with CLL and has demonstrated promising biologic and clinical activity [169].

It is noteworthy that, although the results from preclinical and early clinical studies of these small molecule inhibitors of Bcl-2 family proteins are very encouraging, their clinical usefulness has yet to be formally proved in phase III trials. Furthermore, the mechanisms of killing of leukaemia cells by some inhibitors are not always compatible with that of apoptosis, thus calling into question the mode of cell death induced by such inhibitors. For example, whilst ABT-737 failed to kill cells derived from mouse deficient of both Bax and Bak, a result consistent with activation of apoptosis by mitochondrial pathway, obatoclax was still able to do so [170, 171]. This suggests that obatoclax targets other protein(s) which induce cell death by mechanisms other than the intrinsic death pathway.

6. Concluding remarks

The complexity of GC action and resistance in malignant lymphocytes is evident from the diverse mechanisms responsible for GC resistance in different lymphoid malignancies. Since resistance to GCs limits their therapeutic application, it is

important to understand the mechanisms responsible for GC action and resistance so that rational strategies to overcome resistance can be developed. Significant progress has been made over the last few years in advancing our understandings of these concepts. However, there is much that we still do not understand, and further studies are therefore required.

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