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FOCUSSED RESEARCH REVIEW

Non-genomic events determining the sensitivity of hemopoietic malignancies to glucocorticoid-induced apoptosis

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Abstract Glucocorticoid (GC) hormones have been introduced as therapeutic agents in blood cancers six decades ago. The effectiveness of GC treatment stems from its ability to induce apoptotic death of hemopoietic cells. A major impediment in GC therapy is the acquisition of resistance to the drug upon repeated treatment. In addition, some blood cancers are a priori resistant to GC therapy. Usually, resistance to GC correlates with poor prognosis. Albeit the wide use of GC in clinical practice, their mode of action is not fully understood. The cellular response to GC is initiated by its binding to the cytosolic GC receptor (GR) that translocates to the nucleus and modulates gene expression. However, nuclear activities of GR occur in both apoptosis-sensitive and apoptosis-resistant cells. These apparent controversies can be resolved by deciphering non-genomic effects of GCs and the mode by which they modulate the apoptotic response. We suggest that non-genomic consequences of GC stimulation determine the cell fate toward survival or death. Understanding the cellular mechanisms of GC apoptotic sensitivity contributes to the development of new modalities for overcoming GC resistance.

Keywords Glucocorticoids · Hemopoietic tumors · Mitochondria · BIM · Glycogen synthase kinase 3 · CITIM 2013

Introduction

Glucocorticoid (GC) hormones are commonly used in the treatment for hemopoietic malignancies, due to their ability to induce apoptosis of malignant lymphoid cells. However, a major obstacle to GC-based therapy is the emergence of resistant cells that no longer respond to GC with apoptotic death. Even though GCs have been used in the treatment for leukemias and lymphomas for more than half a century, the mechanisms underlying their apoptogenic activity remain obscure.

So far, most studies on cellular responses to GCs have focused on their genomic effects. The reason for that lies in the documented ability of GC to translocate the GC receptor (GR) to the nucleus, where it modulates transcription of some 300 genes through transactivation or transrepression [1–3]. However, only few of the GC-affected genes, e.g., GR and BIM, have been ascribed to apoptosis, and the mere expression of these gene products is insufficient to induce apoptotic death [4]. It is thus conceivable that additional postgenomic effects operating in lymphoid cells that are sensitive to GC-induced apoptosis might be accountable for the apoptogenic activities of these hormones. In the present article, we shall review some of the non-genomic functions of GC that are associated with the progression of the death response and their relevance in the context of GC-based therapy.

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GC-induced apoptosis in lymphoid cells: role of the mitochondria

Almost all cells of our body express GR and are therefore affected by GC in various ways. However, GC-induced apoptosis is restricted to lymphoid cells, whether normal or malignant. What is the molecular basis for this selectivity? The answer begins to unfold by understanding the consequences of GC binding to its receptor. Normally, the GR is sequestered in the cytosol by a multi-subunit complex of heat shock proteins [5]. Upon GC binding the complex dissociates, setting the GR free to undergo dimerization and phosphorylation before its translocation to the nucleus. These molecular events occur in both apoptosis-sensitive and apoptosis-resistant cells and, thus, cannot be the sole basis for GC-induced apoptosis. We therefore hypothesized that non-genomic effects restricted to lymphoid cells are involved in determining the selectivity of the apoptotic response to GCs. The first validation of our hypothesis emerged when we followed the intracellular trafficking of the GR following stimulation by the synthetic GC dexamethasone (DEX). We detected a mitochondrial localization signal (MLS) within the ligand-binding domain of the GR, which enables its trafficking to the mitochondria in sensitive lymphoid cells only [6, 7]. Mitochondrial translocation of the GR reduces the outer membrane potential, thereby allowing release of cytochrome *c* and Smac/DIABLO [7]. This is a crucial step in activating the intrinsic mitochondrial apoptosis pathway, which occurs in lymphoid cells only, making them sensitive to GC-induced apoptosis.

In this regard, it should be stressed that translocation of GR to the mitochondria is necessary but not sufficient for GC-induced apoptosis. The reason for this is the expression of mitochondrial anti-apoptotic proteins, such as the BCL2 family proteins and the voltage-dependent anion channel (VDAC), that stabilize the outer membrane and offset the pro-apoptotic activity of GR [8]. These proteins have to be deactivated before the GR can advance the intrinsic apoptotic pathway. This step of apoptotic sensitization is regulated through phosphorylation enabled by the cellular kinome as outlined below.

GC-induced apoptosis in lymphoid cells: role of the kinome

Studies in our laboratory have indicated that glycogen synthase kinase 3 (GSK3) and the pro-apoptotic BH3-only BCL2-like protein (BIM) are key protein kinases involved in the GC-induced apoptotic pathway. The role of GSK3 has been demonstrated using specific inhibitors of GSK3, which blocked DEX-mediated apoptosis in sensitive cells [9]. Likewise, a kinase-inactive, dominant negative GSK3 inhibited DEX-induced apoptosis [9]. GSK3 is active in GC-sensitive cells but not in GC-resistant ones as its phosphorylation level is low in the former and high in the latter (unpublished data). GSK3 is further activated upon treatment with DEX. Our investigation indicated that GSK3α is part of the cytosolic multi-subunit complex of GR in unstimulated cells [9]. Once GC binds to the GR, GSK3α dissociates from the complex and exerts its kinase activity alongside GSK3β [9].

The other protein kinase involved is BIM, whose expression is upregulated by GR translocation to the nucleus [10, 11]. Albeit BIM does not pose GC response elements (GREs) in the promoter [10, 12], its expression is upregulated by FOXO3a transcription factor (TF) [13, 14] that responds to GC with upregulated synthesis [15, 16]. Upregulation of BIM is crucial for conferring GC-induced apoptosis in cells that display low basal levels of this protein [17, 18]. However, some GC-sensitive cells express a sufficient amount of BIM that is not further upregulated by DEX [8, 19, 20]. Moreover, upregulation of BIM per se is insufficient to trigger apoptosis as it has to be posttranslationally activated [20]. Indeed, shortly after exposure to DEX, GSK3α and β transiently interact with BIM and trigger its apoptotic activity [9]. BIM mediates BAX and BAK oligomerization, either directly by interaction with BAK and BAX or indirectly by neutralization of BCL2 family proteins that inhibit oligomerization of BAK and BAX [21]. In unstimulated cells, the inactive pro-apoptotic BAK is located in the outer mitochondrial membrane, forming complexes with anti-apoptotic proteins (such as BCL2 and BCL-XL), while BAX is detected in the cytosol [21]. BAX and BAK are crucial pro-apoptotic proteins involved in the formation of the mitochondrial permeability transition pore that initiates the irreversible step of the apoptotic process by enabling release of cytochrome *c* and other pro-apoptotic proteins [22–24]. Hence, activation of BIM by GSK3 relieves the anti-apoptotic effect of BCL2 on GC-induced apoptosis. Furthermore, GSK3β directly phosphorylates BAX at Ser163 and promotes its mitochondrial localization [25], thereby advancing the apoptotic response. In addition, GSK3β phosphorylates VDAC, which prevents its binding to hexokinase II (HKII) [26]. HKII is abundantly expressed in tumor cells since they are highly glycolytic. Avoiding HKII from binding to VDAC potentiates chemotherapy-induced cytotoxicity [26]. Indeed, overexpression of HKII inhibits DEX-induced apoptosis [27] and interferes with the ability of BAX to bind to mitochondria and induce the release of cytochrome *c* [28]. Thus, GC-induced phosphorylation of VDAC and BAX by GSK3 and its interaction with BIM fine-tunes the apoptotic threshold. GSK3 contributes to the activation of the intrinsic apoptotic pathway by deactivating BCL2, VDAC, and HKII while activating BIM, BAX, and BAK, thus allowing mitochondrial GR to advance cellular death.

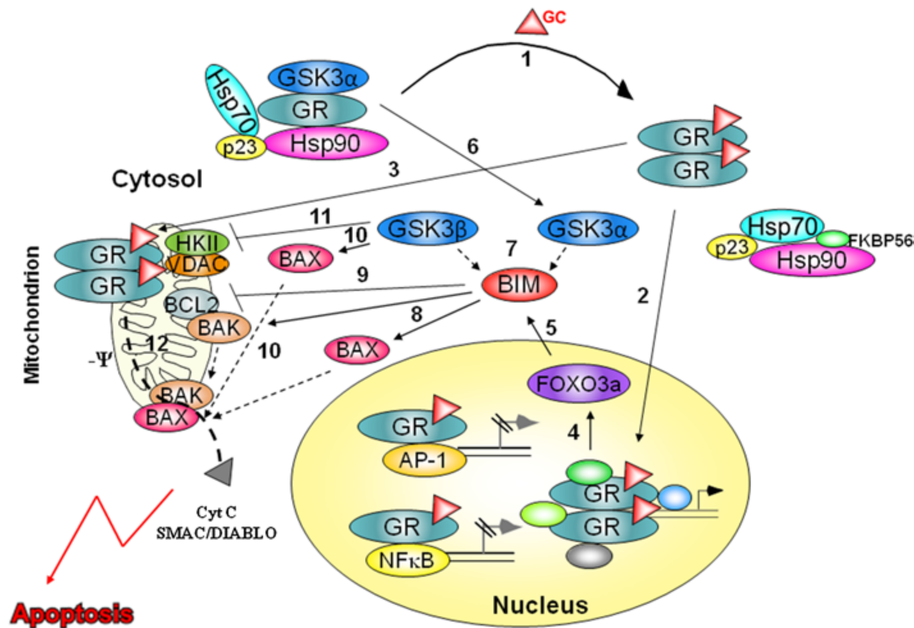

Fig. 1 The consequences of GC stimulation in GC-sensitive cells. In the absence of GC, GR and GSK3α associate in a large heteromeric complex. Upon GC binding, GR dissociates from the complex and undergoes dimerization and phosphorylation (*1*). Dimerized GR translocates to both nucleus (*2*) and mitochondria (*3*). In the nucleus, GR transactivates and transrepresses multiple genes. Of special note is FOXO3a TF (*4*) which upregulates BIM expression (*5*). In addition to GR, GSK3α also dissociates from the heteromeric complex (*6*) and, following immediate interaction with GSK3β, binds BIM to promote its pro-apoptotic activity (*7*). Upregulated and activated BIM induces BAX and BAK oligomerization (*8*) and inhibits BCL2 (*9*). GSK3 phosphorylates BAX and promotes its mitochondrial translocation (*10*). Concomitantly, it phosphorylates VDAC and inhibits its subsequent association with HKII, thus enabling reduction in mitochondrial membrane potential (*11*) by GR (*12*). Reduction in mitochondrial membrane potential along with oligomerization of BAX and BAX results with release of cytochrome *c* and Smac/DIABLO that initiates the apoptotic process

The model

Based on these studies, we propose the following model: In unstimulated cells, GSK3α associates with the GR multi-subunit complex [9] (Fig. 1). When a ligand is bound to the GR, GSK3α dissociates and GR dimers are trafficking to the nucleus and mitochondria (Fig. 1). At the same time, the two isoforms of GSK3 interact with BIM [9], which is upregulated by nuclear GR through FOXO3a TF [13–16] (Fig. 1). BIM inhibits BCL2 and activates BAX and BAK [21]. Furthermore, GSK3 directly activates BAX by promoting its mitochondrial localization [25] and inhibits VDAC association with HKII [26]. These interactions enable mitochondrial GR to reduce the outer membrane potential followed by the release of cytochrome *c* and Smac/DIABLO which initiates the apoptotic process [7] (Fig. 1).

Relevance to therapy

Given this knowledge, we could ask several questions related to therapy of leukemias and lymphomas by GC hormones, such as prednisone and DEX. Why some of these cancers are resistant a priori to hormonal treatment? And why sensitive leukemias and lymphomas gradually develop resistance to the GC treatment? The reason can be attributed to the dysfunction of GSK3 and BIM. Activity of GSK3 is regulated by the PI3K–AKT axis, which inactivates GSK3 by phosphorylation at Ser21 and Ser9 on GSK3α and GSK3β, respectively [29, 30]. One mode by which a resistant phenotype is attained is through hyperactivity of AKT. GC-resistant cells display highly activated AKT that leads to GSK3 inactivation [9]. In these cells, the GR does not translocate to the mitochondria [6]. AKT antagonizes GC-induced apoptosis in follicular lymphoma [31], multiple myeloma [32], peripheral T cells [33], thymoma [9], T-acute lymphoblastic leukemia [9], and Burkitt's lymphoma (unpublished data). Therefore, GC resistance may be reversed by specific AKT inhibitors, such as *wortmanin* and *staurosporine* [8, 9]. These drugs, when given together with DEX, retain the sensitivity of leukemia cells to apoptosis and prolong the response of the cells to GC [8, 9]. Indeed, apoptosis conferred by *wortmanin* or *staurosporine*, when combined with DEX, is prevented by inhibition of GSK3 [9] (unpublished data). In addition to their effect on GSK3, PI3K/AKT inhibitors also mediate BIM upregulation through activation of FOXO3a [13, 34]. AKT phosphorylates FOXO3a on Thr32, Ser253, and

Ser315, a process that causes its binding to the 14-3-3 proteins. This interaction leads to their immediate export from the nucleus followed by inhibition of FOXO3a-dependent transcription [34]. Indeed, FOXO family proteins regulate cell survival by transcriptionally modulating the expression of death receptor ligands and BIM [13]. Hence, inhibition of PI3K/AKT signaling relieves the inhibitory phosphorylation from FOXO3a, which upregulates expression of BIM.

We have also found that inhibition of PI3K/AKT mediates Nur77 activation [8, 35] through inhibition of its proteasomal degradation [8]. Nur77 is a transcription factor controlled by external stimuli, but it is not expressed in resting cells. Nur77 mRNA is upregulated in both B [36] and T [37, 38] lymphocytes following activation by antigen–receptor ligation. It has been implicated in negative selection of T cells [39], whose pro-apoptotic action cannot be offset by BCL2 [40, 41]. Nuclear Nur77 may cause apoptosis by upregulating pro-apoptotic proteins, such as FasL and TRAIL [41, 42]. Nur77 also acts at the mitochondria by binding to BCL2 and converting it into a pro-apoptotic protein [40, 41]. AKT directly phosphorylates Nur77 in its DNA-binding domain, resulting in reduced Nur77 DNA binding and transcriptional activity [35]. In addition, AKT stimulates Nur77 association with 14-3-3 protein by phosphorylating Nur77, a process that inhibits Nur77 activity [43]. Furthermore, AKT phosphorylation of Nur77 blocks its mitochondrial targeting and association with BCL2 [44]. Indeed, AKT mediates reduction in activation-induced cell death of T cell hybridomas by inhibition of Nur77 [43]. We demonstrated that in low-BCL2-expressing cells, inhibition of PI3K/AKT causes Nur77 to act mainly at the nucleus as a promoter of GC-induced apoptosis. However, in high-BCL2-expressing cells, when PI3K/AKT is inhibited, Nur77 acts at both the nucleus and mitochondria, since BCL2 entraps some Nur77 particles and avoids them from translocating to the nucleus (unpublished data). Inhibition of Nur77 in BCL2-positive or BCL2-negative GC-resistant cells avoids PI3K/AKT inhibitors from sensitizing these cells to GC-induced apoptosis [8] (unpublished data). Nur77 is not affected by DEX treatment in both GC-sensitive and GC-resistant cells; thus, Nur77 upregulation is not directly involved in advancing GC-induced apoptosis but acts to overcome GC resistance. Finally, the PI3K/AKT inhibitor *staurosporine* slightly induces GR translocation to the mitochondria upon DEX treatment of GC-resistant cells [8]. However, the level of mitochondrial GR in such cells is far below that observed in GC-sensitive ones. Hence, it is unlikely that this is the major mode by which *staurosporine* sensitizes cells to GC-induced apoptosis.

In summary, GC-resistant cells are distinguished from sensitive ones by three parameters as follows: (1) GSK3α is not bound to their cytosolic unstimulated GR, (2) upon stimulation with GC, their GR does not translocate to the mitochondria, and (3) their PI3K/AKT signaling pathway is highly activated. They express high level of anti-apoptotic proteins belonging to the BCL2 superfamily (Fig. 2). AKT inactivates FOXO3a by phosphorylation on Thr32, Ser253, and Ser315 [34], and as a consequence, BIM upregulation is inhibited (Fig. 2). AKT also inactivates GSK3 and Nur77 by inhibitory phosphorylation on Ser21/9 and Ser350, respectively [30, 35] (Fig. 2). BIM and GSK3 are essential for GC-induced apoptosis, and therefore, their inhibition by AKT confers GC resistance. In addition, resistant cells usually express high levels of BCL2 superfamily proteins. Such proteins further antagonize GC-induced apoptosis by inactivating BAX and BAK, a process that can be overcome by hyperexpression of BIM [45] (Fig. 2). Forced inhibition of PI3K/AKT signaling relieves the inhibitory phosphorylation from FOXO3a, GSK3, and Nur77 but rarely induces mitochondrial GR translocation (Fig. 2). As a consequence, GC-mediated FOXO3a upregulation is not inhibited by AKT, and therefore, it is free to induce BIM transcription. Active GSK3 further activates BIM and BAX and inhibits many survival signaling pathways, such as β-catenin, VDAC, and HKII. Finally, activated Nur77 translocates either to the nucleus, where it upregulates transcription of pro-apoptotic genes such as FasL and TRAIL [41, 42], or to the mitochondria, where it converts anti-apoptotic BCL2 to a pro-apoptotic protein [40]. Upregulated and activated BIM, along with inhibition of BCL2 and VDAC, positively regulates BAK and BAX, thereby enabling the advancement of the apoptotic pathway.

Conclusions

We have defined two levels at which GC-induced apoptosis of leukemia and lymphoma cells is regulated. Receptor translocation to the mitochondria distinguishes between sensitive lymphoid cells and other cells expressing GR but inherently resistant to GC-induced apoptosis. Mitochondrial GR is mandatory but not sufficient to induce apoptosis. Concomitantly, the cell kinome is playing a crucial role in advancing the intrinsic apoptotic pathway initiated by mitochondrial GR. In this regard, activation of the protein kinases GSK3 and BIM is essential for the GC-mediated apoptotic death response.

Each level can become a subject for intervention to prolong the sensitivity of hemopoietic malignant cells to GC therapy or even resensitize fully resistant cells to respond. We have shown earlier that *staurosporine* induces GSK3 activation, as well as upregulation of Nur77 and BIM. By these virtues, *staurosporine* can turn resistant cells into sensitive ones. Specific inhibitors of PI3K and AKT, such as *wortmanin*, help in keeping GSK3 active and defer

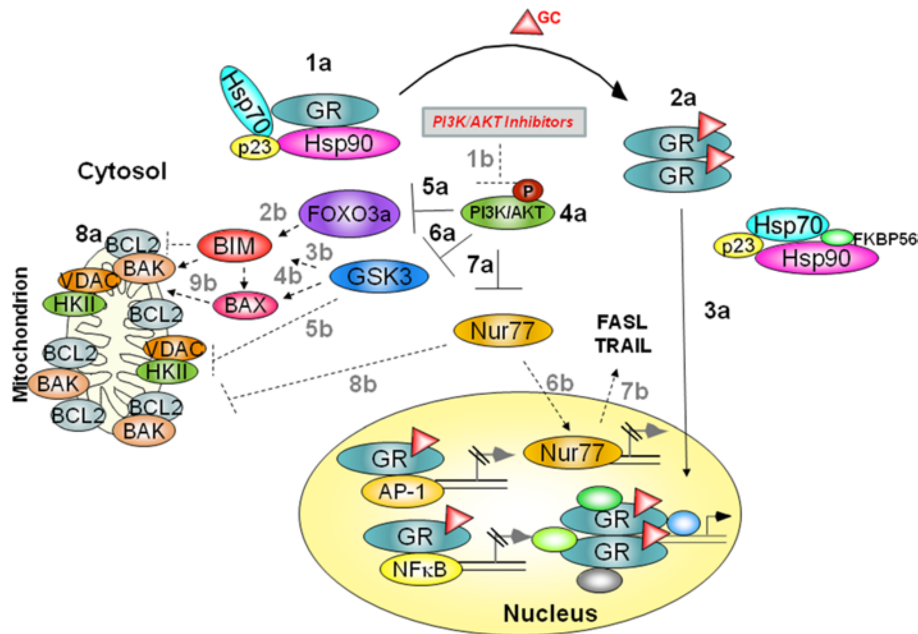

Fig. 2 **a** The consequences of GC stimulation in GC-resistant cells. In the absence of GC, GR is sequestered in a large heteromeric complex (*1a*). Upon GC exposure, GR dissociates from the complex and undergoes dimerization and phosphorylation (*2a*). Dimerized GR translocates to nucleus, but not to mitochondria (*3a*). In addition, BIM does not respond with upregulation as AKT is highly activated (*4a*). Activated AKT phosphorylates and inactivates FOXO3a (*5a*). AKT also inactivates GSK3 and Nur77 by inhibitory phosphorylations (*6a*–*7a*). Moreover, resistant cells express high levels of anti-apoptotic BCL2 superfamily proteins (*8a*), conferring a resistant phenotype on the cells. **b** Overcoming GC resistance by PI3K/AKT inhibitors: Inhibition of PI3K/AKT signaling by *wortmanin* or *staurosporine* (*1b*) relieves the inhibitory phosphorylation from FOXO3a, GSK3, and Nur77. GC-mediated FOXO3a upregulation is not inhibited by AKT, which is free to induce BIM transcription (*2b*). Non-phosphorylated GSK3 further activates BIM (*3b*) and BAX (*4b*) and inhibits VDAC association with HKII (*5b*). Finally, Nur77 is upregulated and translocates to the nucleus (*6b*), where it upregulates pro-apoptotic genes, such as FasL and TRAIL (*7b*). In addition, Nur77 translocates to mitochondria and converts anti-apoptotic BCL proteins to pro-apoptotic ones (*8b*). Activated BIM, accompanied with pro-apoptotic BCL2, activates BAX and BAK (*9b*), thus enabling the advancement in the apoptotic pathway

the emergence of cells that become apoptotic resistant in response to GCs, thus adding a new opportunity for retaining the apoptotic response to CG hormones. Further studies may identify additional protein kinase-specific inhibitors that can effectively sensitize hematopoietic cancer cells to GC-induced apoptosis.

Glucocorticoid-based therapy has been practiced for more than half a century and seems to remain as a principal tool in the management of blood malignancies. It is thus highly desirable to search for treatment modalities that maintain and prolong the apoptotic response to the hormonal treatment. To this end, combining GC therapy with protein kinase inhibitors that offset GC resistance may be a promising approach.

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Conflict of interest The authors declare that they have no conflict of interest.

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