MECHANISMS OF STEROID ACTION AND RESISTANCE IN INFLAMMATION

Resistance to glucocorticoid-induced apoptosis in lymphoblastic leukemia

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Abstract

Glucocorticoid (GC) resistance is a phenomenon of major significance in a number of clinical situations, including the therapy of lymphoid malignancies. Resistance may concern all, or just selected, GC effects, it may be absolute or just reflect a state of reduced sensitivity and, clinically relevant, be reversible or irreversible. Numerous molecular mechanisms can be envisaged acting either 'upstream' in the GC-triggered signaling pathway, i.e. at the level of the GC receptor (GR), or 'downstream' at the level of the GC-regulated genes responsible for individual GC effects.

In lymphoid malignancies, GCs have anti-leukemic effects through the induction of apoptosis and/or cell cycle arrest. In this condition evidence for only a small number of mechanisms for GC resistance has been provided, mostly at the level of the GR. Herein, we review reports and hypotheses regarding 'upstream' and 'downstream' mechanisms for GC resistance in lymphoblastic leukemia and present an *in vitro* GC resistance model that might allow identification of resistance mechanisms.

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Introduction

Because of their pleiotropic effects, glucocorticoids (GCs) are among the most frequently prescribed drugs. In lymphoid malignancies, these hormones and their analogs have anti-leukemic actions leading to the induction of apoptosis and/or cell cycle arrest (for recent reviews see King & Cidlowski 1998, Thompson 1998, Kofler 2000, Planey & Litwack 2000, Distelhorst 2002, Greenstein *et al.* 2002). However, some tumors do not respond to these physiological drugs *a priori* (primary resistance) and those which do almost always develop resistance during therapy (secondary resistance) (Kaspers *et al.* 1994, Moalli & Rosen 1994). In spite of its clinical significance (Klumper *et al.* 1995), the molecular basis of GC resistance is still poorly understood. The term 'GC resistance', both in general and in lymphoid malignancies in particular, is used with quite different meanings. Resistance can occur on the level of the entire organism, as in primary cortisol resistance, or affect the descendants of a particular cell clone, as in lymphoblastic leukemia. GC resistance can concern all, a few, or just single effects. It may be reversible, as during lymphocyte differentiation where sensitivity to GC-

induced apoptosis varies dramatically (Ashwell *et al.* 2000, Winoto & Littman 2002), or irreversible as in the case of GC receptor (GR) mutations. It can be absolute, i.e. GC concentration-independent, or relative, i.e. reflecting reduced sensitivity, and apply to some but not other GC analogs. Such distinctions are clinically relevant as higher-dose GC treatment abrogated the effect of relative drug insensitivity and low GR levels in childhood acute lymphoblastic leukemia (ALL) (Schwartz *et al.* 2001).

Regarding possible molecular mechanisms, an almost endless number can be envisaged along the signal transduction pathways triggered by GC (Fig. 1). 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. The latter interfere with individual GC responses, such as induction of apoptosis. Below, we discuss up- and downstream mechanisms for resistance to GC-induced apoptosis with particular emphasis on human lymphoid malignancies and conclude by presenting an *in vitro* model for GC resistance in childhood ALL and first evidence for possible resistance mechanisms derived therefrom.

Figure 1 Principal mechanisms of GC resistance. Possible resistance mechanisms are organized along the GC signaling pathway: 'upstream' mechanisms include (1) 'pre-receptor defects', (2) structural and regulatory abnormalities in GR expression, and (3) deficiencies in GR-associated proteins, and may affect most, if not all, GC responses. 'Downstream' mechanisms encompass (4) defects in components of the specific response pathway or (5) cross-talk from other signaling pathways that interfere with and antagonize a given GC response. Abbreviations: 11β-OHSD, 11β-hydroxysteroid dehydrogenase; C, chaperones; GC, glucocorticoid; GR, glucocorticoid receptor; Pgp, P-glycoprotein; R, ribosome.

GC resistance in lymphoid malignancies – 'upstream mechanisms'

At present, convincing evidence for a causative role in resistance to GC-induced apoptosis has mostly been provided for 'upstream mechanisms'. Since GC resistance results from a selection process for both survival and proliferation, this might be taken as evidence that the GR constitutes the only common, and hence most sensitive, step in the respective pathways. Alternatively, it may simply reflect experimental biases and/or our ignorance concerning critical downstream components.

Pre-receptor mechanisms

Overexpression of the *mdr-1* gene-encoded P-glycoprotein, an A/B/C transporter that 'pumps' various GC analogs out of the cell, has been suggested to account for cross-resistance resulting after exposure to combination chemotherapy (Arceci 1993, Bourgeois *et al.* 1993, Gottesman *et al.* 2002). In addition to affecting apoptotic

responses to other agents as well, this form of GC resistance is characterized by its sensitivity to P-glycoprotein inhibitors, like verapamil or cyclosporin A, and its differing efficiency towards various GC analogs (Karssen *et al.* 2001). A second pre-receptor mechanism is more theoretical at present and concerns expression of GC-metabolizing enzymes such as 11β-hydroxysteroid dehydrogenase that converts cortisol into inactive cortisone (Funder 1996, Seckl & Walker 2001).

GR gene mutations and polymorphisms

Numerous loss-of-function mutations in the GR gene have been observed in GC-resistant human ALL cell lines (Ashraf & Thompson 1993, Powers *et al.* 1993, Strasser-Wozak *et al.* 1995, Hala *et al.* 1996, Chen *et al.* 1997). Interestingly, these and the mutations observed in primary cortisol resistance (Chrousos *et al.* 1993, Werner & Bronnegard 1996) and in mouse leukemia (see reviews in the Introduction), concern the DNA- and ligand-binding domains, whereas the large N-terminal region containing

Figure 2 The human GR gene (NR3C1) and GR variants. The top graph depicts the GR gene on chromosome 5q31/32, with boxes and lines corresponding to exons and introns respectively (not to scale). Alternative splice variants are indicated. Note that the GR gene has three published (Breslin *et al.* 2001) and at least four additional untranslated exons 1 (E Presul & R Kofler, unpublished observations) whose significance is unknown. The graphs below represent various GR isoforms resulting from alternative splicing (GR-α, -β, -γ and GR-A), polyadenylation (GR-P/GR-δ), and translation initiation (GR-B). A/B, C, D and E refer to protein regions of nuclear receptors, the numbers denote amino acid positions (Laudet & Gronemeyer 2002). AF, sequences implied in transactivation; NLS, nuclear localization sequence; LBD, ligand-binding domain.

one of the transactivating domains is hardly ever affected, possibly reflecting the limited sequence requirements of transactivating domains. Whether GR gene mutations are the major GC resistance mechanism *in vivo* as well is still unresolved. From a theoretical stand-point, the combination of chemotherapy with its mutagenic potential with GC might indeed favor the development of, and selection for, GR mutations, as has been shown *in vitro* (Palmer *et al.* 1992). However, in one study, no evidence for mutations in the DNA- and ligand-binding domains was observed in 22 chronic lymphatic leukemia patients subjected to combination chemotherapy (Soufi *et al.* 1995). In child-

hood ALL, evidence for GR mutations has been provided in one case (Hillmann *et al.* 2000). Whether the two known GR polymorphisms, i.e. N363S and R23K, affect GC sensitivity is still controversial (Koper *et al.* 1997, Huizenga *et al.* 1998, De Lange *et al.* 1999).

GR variants

As outlined in Fig. 2, a number of GR variants resulting from alternative splicing, polyadenylation or translational initiation have been described. Two of them (GR-P/GR-δ and GR-A) were originally detected in a

GC-resistant myeloma cell line (Moalli *et al.* 1993). GR-P has subsequently been observed in a number of hematopoietic and other malignancies as well as in normal lymphocytes (Moalli & Rosen 1994, Krett *et al.* 1995, De Lange *et al.* 2001). How it might affect GC sensitivity has remained controversial with reports suggesting that it might contribute to a resistant phenotype (Gaitan *et al.* 1995, Krett *et al.* 1995) and others providing evidence that GR-P increased the activity of a concomitantly expressed wild type GR (De Lange *et al.* 2001). Another splice variant, termed GR- β and reportedly encoding a dominant negative GR protein (De Castro *et al.* 1996, Oakley *et al.* 1999), has been implicated in various forms of GC resistance (Leung *et al.* 1997, Shahidi *et al.* 1999, Strickland *et al.* 2001) including that induced by tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 in CEM-C7 T-ALL cells (Webster *et al.* 2001). Whether expression of this isoform might account for GC resistance in patients with lymphoblastic malignancies is still unclear: Longui *et al.* (2000) concluded that the combination of low GR- α and normal-to-high GR- β expression in leukemic lymphoblasts might represent one of the mechanisms for reduced GC sensitivity in ALL, particularly T-ALL. De Lange *et al.* (2001) found little, if any, expression of GR- β in various hematopoietic tumors. In concert with reports that failed to discover dominant negative activity of GR- β (Hecht *et al.* 1997, De Lange *et al.* 1999), this argued against a critical role of the GR- β isoform in GC resistance in leukemia. Two further GR variants have been described: the splice variant GR- γ , with an additional arginine in position 450A and about 50% less transactivation ability than GR- α (Rivers *et al.* 1999), and GR-B that lacks the N-terminal 16 amino acids because of alternative translation initiation and which is nearly twice as effective as the longer GR- α species in gene transactivation, but not in transrepression (Yudt & Cidlowski 2001). Whether expression of these variants affects sensitivity to GC-induced apoptosis in lymphoid malignancies is unknown.

Insufficient GR expression

GR expression levels have been correlated with GC sensitivity in numerous experimental systems (Bellingham *et al.* 1992, Pazirandeh *et al.* 2000, Reichardt *et al.* 2000). As long as 30 years ago, the possible importance of insufficient GR expression in GC resistance in late stage leukemia was suggested by E Brad Thompson and co-workers (Lippman *et al.* 1973). More recently, GR levels in childhood ALL correlated with the *in vivo* response to single-agent GC before combination induction chemotherapy (Pui *et al.* 1984), and with disease outcome in a large study including 546 children with ALL (Kato *et al.* 1993). However, such a correlation was not always seen (Csoka *et al.* 1997) and basal expression levels may be only part of the story. A number of reports suggest that GR

auto-induction might be critical for sensitivity to GC-induced apoptosis: early studies have shown a correlation between GC-sensitivity and GR auto-induction in myeloma (Gomi *et al.* 1990) and lymphoblastic leukemia (Antakly *et al.* 1989, Denton *et al.* 1993, Barrett *et al.* 1996) cell lines. Functional requirement of GR upregulation for GC-induced apoptosis was further shown in elegant experiments in CCRF-CEM T-ALL cells (Ramdas *et al.* 1999). We observed that the GR was one of only eight genes that appeared co-regulated in a comparative expression profiling study with over 7000 genes in proliferating and cell cycle-arrested CEM cells both undergoing GC-induced apoptosis (Tonko *et al.* 2001). Together with the long-known observation, recently supported by expression profiling studies (Obexer *et al.* 2001, Tonko *et al.* 2001), that GC repress a number of metabolic enzymes, as well as general transcription and translation, we put forward the hypothesis that positive GR feed-back regulation maintains these GC effects to an extent not compatible with cellular survival (Kofler 2000). Further experimental evidence for the requirement of a positive GR feed-back loop is presented in the last Section.

GR-associated proteins causing impaired GR function

In the cytoplasm, GR associates with chaperones including various heat shock proteins and immunophilins, which ensure proper folding for ligand binding and may contribute to subsequent nuclear transport (Bohen *et al.* 1995, Pratt *et al.* 1999). In the nucleus, the GR recruits a number of co-factors such as SRC-1, TIF2/GRIP1, CBP/p300, NcoR and SMRT required for its gene regulatory activities (Beato *et al.* 1995, Laudet & Gronemeyer 2002). Mutations in, or abnormal expression of, essential components of the different GR complexes might therefore compromise GR function. As examples, overexpression of the immunophilin RAP46/BAG-1 prevented GC-induced apoptosis in S49 mouse leukemia cells (Schneikert *et al.* 1999) and insufficient SRG3 (Swi-3 related gene) expression has been associated with reduced resistance to GC-induced apoptosis in peripheral mouse T cells (Han *et al.* 2001b). A particular situation concerns GR interactions with other sequence-specific transcription factors, in particular AP-1 (De Bosscher *et al.* 2001, Herrlich 2001) and NF- κ B (Wissink *et al.* 1997, McKay & Cidlowski 1998). These interactions frequently lead to mutual antagonism and overexpression of such factors might therefore interfere with many, if not all, GR-mediated responses. In addition, most of these factors control survival pathways. Thus, the balance between the pro-apoptotic GR and these anti-apoptotic transcription factors may control life or death decisions. The above interactions might therefore lead to upstream as well as downstream interference with GC-induced cell death. Moreover, they might explain GC-apoptosis antagonizing

effects of some lymphokines (see Section on lymphokines below). In the clinical situation, activation of these transcription factors by multiple mechanisms might contribute to GC resistance; however, convincing evidence for such resistance mechanisms is still lacking.

GC resistance in lymphoid malignancies – ‘downstream resistance mechanisms’

Resistance to a particular GC effect, like GC-induced apoptosis, might be caused by defects in downstream components of the specific response pathway or cross-talk from other signaling pathways that interfere with the given GC response.

Defects in GC target genes

Since the GC-regulated genes responsible for apoptosis induction in lymphoid malignancies are still enigmatic, corresponding resistance mechanisms have not been reported and are difficult to predict. A number of GC-regulated genes including *c-myc* (Thulasi *et al.* 1993, Medh *et al.* 2001), *IκB* (Auphan *et al.* 1995, Ramdas & Harmon 1998), *c-jun* (Barrett *et al.* 1996, Zhou & Thompson 1996), and cyclin D3 and *cdk4* (Rogatsky *et al.* 1997, M J Ausserlechner, P Obexer, S Geley & R Kofler, unpublished observations) have been implicated from studies employing human leukemia and osteosarcoma cell lines (these and many other genes have also been reported in rodent cells – see reviews in the Introduction). In some cases, like *c-myc* and cyclin D3 (Löffler *et al.* 1999, M J Ausserlechner, P Obexer, S Geley & R Kofler, unpublished observations), their functional role in leukemia apoptosis has, however, been questioned. Expression profiling studies with GC-treated ALL (Obexer *et al.* 2001, Tonko *et al.* 2001, Yoshida *et al.* 2002) or myeloma (Chauhan *et al.* 2002) cell lines have revealed a considerable number of additional candidate genes. However, unequivocal proof for a functional role in GC-induced apoptosis of human leukemia has not been provided for any one of them, nor is there a single example of GC resistance caused by mutation in, or deficient GC regulation of, any one of these genes in human lymphoid malignancies.

GC resistance as result of inhibitory cross-talk at the effector level

Resistance to GC-induced apoptosis might further result from interfering with the apoptotic effector machinery, e.g. by activation of survival pathways. This mechanism, however, might only work if GC triggers cell death by activating the apoptotic program without severely damaging the cell. If GC massively damaged the cells (which may be the case after GR auto-induction (Kofler 2000)), interference with the apoptotic machinery without

preventing the primary damage to the cell may not restore long-term survival. In human lymphoid malignancies, two resistance situations have been investigated in detail that might correspond to inhibitory cross-talk mechanisms, i.e. overexpression of anti-apoptotic Bcl-2 family members and resistance mediated by lymphokines.

GC resistance and the Bcl-2 rheostat Although it is unresolved whether components of the Bcl-2 rheostat (Oltvai & Korsmeyer 1994) are regulated by GC in human lymphoid malignancies (GCs repress Bcl-2 for instance in human osteosarcoma (Rogatsky *et al.* 1999) and induce the BH3-only molecule Puma/bbc3 in mouse thymocytes (Han *et al.* 2001a)), it is clear that anti-apoptotic Bcl-2 members exert inhibitory effects upon GC-induced cell death in such cells (Brunet *et al.* 1998, Hartmann *et al.* 1999). However, these effects are transient in the continuous presence of GC, and Bcl-2 does not prevent cell cycle arrest (Hartmann *et al.* 1999), altogether limiting its potential in a selection process that requires both proliferation and survival. In any case, expression of anti-apoptotic Bcl-2 members is frequent in leukemic cell lines and primary cells from patients prior to, and particularly after combination chemotherapy, and has been associated with resistance to chemotherapeutic drugs and GC (Smets & Van den Berg 1996, Salomons *et al.* 1997).

Resistance through lymphokines – activation of survival pathways? Autocrine or paracrine secretion of lymphokines with subsequent activation of survival pathways has been implicated in GC resistance in numerous systems. In multiple myeloma, activation of the focal adhesion tyrosine kinase RAFTK/Pyk2 was reported to be required for GC-induced apoptosis (Chauhan *et al.* 1999), and IL-6, a known autocrine growth and survival factor for such cells (Frassanito *et al.* 2001), blocked both RAFTK activation (via the protein tyrosine phosphatase 2, SHP2) and GC-induced apoptosis (Chauhan *et al.* 2000). IL-6 has further been suggested to prevent apoptosis in this system by activating PK-B/Akt, a potent survival kinase (Hideshima *et al.* 2001). IL-2 and IL-4 have been reported to activate the survival transcription factor NF-κB by repressing its inhibitor IκB (Xie *et al.* 1997), thereby counteracting the proposed apoptosis-inducing induction of IκB by GC (Auphan *et al.* 1995, Ramdas & Harmon 1998). In addition, NF-κB may act as ‘upstream’ inhibitor of GC responses by binding to and inhibiting the GR (McKay & Cidlowski 1998). Similarly, activation of STAT6 with subsequent inhibition of the GR has been shown to be responsible for GC resistance after IL-4 exposure (Biola *et al.* 2000). Since TNF-α and IL-1 seem to interfere with GC-induced apoptosis by changing the GR-α/-β ratio (Webster *et al.* 2001), lymphokines may, in addition to activating survival pathways, frequently induce ‘upstream’ GC resistance.

Figure 3 GC resistance is associated with impaired GR and/or GILZ induction. Ten GC-sensitive (open triangles) and 41 GC-resistant (black circles) subclones of the GC-sensitive CCRF-CEM-C7H2 human T-ALL cell line were treated with 10^{-7} M dexamethasone for the indicated times and their RNA subjected to quantitative 'real time' PCR to assess the levels of GR and GILZ in relation to TATA-box-binding protein used as a control. Note that the scale of the x-axis is different in the three plots for better data visualization. After 24 h dexamethasone exposure all GC-sensitive, but none of the GC-resistant, cells shift to the top right quadrant. All cells (including the GC-sensitive ones) are alive at 24 h, apoptosis starts around 36 h in GC-sensitive cells.

An *in vitro* model for GC resistance in ALL

To address the potential of developing resistance mechanisms in ALL cells, we generated panels of GC-resistant and -sensitive subclones of the CCRF-CEM-C7H2 cell line by limiting dilution cloning in the presence and absence of 10^{-7} M dexamethasone. DNA finger-printing with 16 short tandem repeat (STR) markers showed that many of these lines can be distinguished by one or two altered STRs documenting their independent subclone nature. Although intended to be used for extensive comparative expression profiling, we first determined the expression of GR mRNA in these cell lines prior to, and 6–8 h and 24 h after exposure to 10^{-7} M dexamethasone using quantitative 'real time' RT-PCR. We further quantified expression levels of GILZ, a GC-induced leucine zipper protein implicated in the antagonistic effect of GC on T-cell receptor-induced apoptosis in thymocytes (D'Adamio *et al.* 1997, Riccardi *et al.* 1999). As depicted in Fig. 3, all GC-sensitive lines, like parental C7H2 cells, markedly induced both GR and GILZ mRNAs after 6–8 h and 24 h. In sharp contrast, 37 of 41 resistant lines failed to regulated GR and GILZ to the same extent as the sensitive lines. Since all but five resistant cell lines showed detectable induction of GR and/or GILZ (albeit to lower levels than the GC-sensitive lines), we concluded that these lines expressed functional GR. Thus, at least in this model system, GC resistance was most frequently associated with a failure to auto-upregulate GR expression. The significance of impaired GILZ induction, which was also closely associated with GC resistance, is currently being investigated.

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