Glucocorticoid receptor-mediated apoptosis: mechanisms of resistance in cancer cells

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Abstract
Glucocorticoids (Gcs) are commonly used to treat patients suffering from a wide range of cancers. Their main therapeutic role is based on Gc receptor (GR)-mediated mechanisms that trigger cell death but this varies depending on the cancer type. This review aims to provide an overview of the mechanisms of Gc-induced cell death and more importantly the changes in GR that lead to resistance to Gc treatment in cancer. The three main cancer types, which are susceptible to Gc resistance and therefore loss of Gc-induced apoptotic effects, are acute lymphoblastic leukaemia, osteosarcoma and small-cell lung carcinoma. A common theme is the loss of GR function and/or a downregulation of GR expression which leads to failure of the death-inducing effects of Gcs. Loss of GR function is attributed to mutations in the GR gene, and in some cases a dominant-negative effect on any functional GR still present. The downregulation of GR expression can be due to decreased GR promoter activation, increased GR promoter methylation or increased expression of alternative splice isoforms of GR that have decreased transcriptional activity. Understanding the mechanisms behind Gc-triggered apoptosis and the resistance to it in these cancer types will help in further refining treatment regimens for patients and will decrease the chance of relapse caused by Gc-resistant cancer phenotypes.

Glucocorticoid treatment of cancer
Glucocorticoids (Gcs) are widely used for the treatment of lymphoid malignancy (Pirotte et al. 1997, Sionov et al. 2008) because of their dramatic effects on cell cycle progression and apoptosis. They are also used as a co-medication in the therapy of solid tumours, either because of their effectiveness in treating the malignancy, or for decreasing oedema, pain, electrolyte imbalance, nausea and emesis or to reduce cytotoxic reactions caused by other treatment regimens (Rutz 2002, Rutz & Herr 2004). The direct effects that Gcs have on cancer cells are varied but detailed studies have enabled a greater understanding of the effects they exert on cell death. Gcs are able to alter signalling in key survival pathways and this can result in reversible growth arrest or cell death in certain cell types.

Endogenous Gcs are involved in a variety of biological processes. In man, Gcs have important effects on metabolism, the immune response, development, the cardiovascular system and reproduction. However, one of the main functions of Gcs is that they reduce inflammatory responses. Because of this, synthetic Gcs are used to treat symptoms of asthma, arthritis and dermatitis. At the cellular level, Gcs impact on processes ranging from proliferation and differentiation to apoptosis. It is this latter action that is important in their use as therapeutic agents in the treatment of cancer.

How do Gcs exert their effects at the cellular level?
Gcs are members of the steroid hormone family, which exert most of their effects by altering transcription of an array of steroid responsive genes. The transcriptional effects are mediated via the Gc receptor (GR), which is a member of the nuclear hormone receptor family.

Gcs bind GR, which then functions as a transcription factor to trigger genomic effects, however there is now evidence of faster effects, mediated non-genomically via membrane-associated receptors and their signalling cascades (Beck et al. 2009, Revollo & Cidlowski 2009). The GR gene generates several different splice and translation protein variants all of which are modular proteins that contain three distinct
functional regions: an N-terminal transactivation domain, a central DNA-binding domain and a C-terminal region that contains the hormone-binding site (Gross et al. 2009).

Genomic actions of Gcs are mediated by nuclear translocation of the GR (Fig. 1). In the absence of Gc ligand, cytoplasmic GR forms a heterocomplex with chaperone proteins which keeps the GR in an inactive state (Cadepond et al. 1991). Upon Gc binding, the GR dissociates from its chaperone proteins, homodimerises via the C-terminal ligand-binding domains (Bledsoe et al. 2002) and translocates to the nucleus. Once in the nucleus, the GR can then act as either a transcriptional activator or a repressor depending on the gene and the cellular environment.

GR dimers are able to bind to the Gc response elements (GREs) present in the promoter regions of the target genes. Once bound to the GRE, the GR recruits co-activator proteins that open the chromatin structure such as histone deacetyltransferases. These include members of the p160 family of proteins (SRC1, PGC1, AIB1, etc.). Upregulation of transcription can usually be observed a few hours after the original Gc signal (Dean & Sanders 1996). GR can also upregulate gene transcription using mechanisms other than classical GREs; GR composite regulatory elements exist where GR interacts with DNA along with other transcription factors such as c-Myb (Geng & Vedeckis 2005) and the GR can also bind to other DNA-bound transcription factors, a process termed ‘tethering’, and modulate their effects as seen with the Gc-mediated regulation of mitogen-activated protein kinases (MAPK)-phosphatase 1 (DUSP1) transcription where the GR effect is modulated by binding to promoter bound C/EBPβ (Johansson-Haque et al. 2008).

Gc bound GR can also repress target genes via similar mechanisms to those used for activation of transcription, i.e. direct DNA binding via negative GREs (nGREs). This leads to recruitment of co-repressor proteins such as histone deacetylases, for example NCoR and SMRT, that ‘close-up’ chromatin to form a structure that does not favour transcription (Perissi et al. 2010). The GR is also able to repress transcription by DNA-based interactions with other transcription factors in composite elements, for example in Gc-mediated repression of the corticotropin-releasing hormone gene (Malkoski & Dorin 1999). Other mechanisms of repression include tethering other DNA-bound transcription factors such as in the repression of NF-κB action (Nissen & Yamamoto 2000) and via competition with a transcriptional activator for DNA-binding sites such as repression of FasL expression by NF-κB (Novac et al. 2006).

Figure 1 Glucocorticoid receptor signalling. The glucocorticoid receptor translocates to the nucleus upon ligand binding where it will act as a homo- or heterodimer with or without other co-factors to either repress or drive transcription of target genes.
Observations from transgenic animals that express a dimerisation-deficient mutation of the GR suggest that a proportion of the effects of Gcs are not modulated by dimerisation-dependent DNA interactions (Reichardt & Schütz 1998). Some of these effects can be explained by the modulation of cytoplasmic pathways via direct protein–protein interactions as shown for example with JNK (Löwenberg et al. 2008, Beck et al. 2009). Gcs exert a number of rapid actions that are independent of the regulation of gene transcription. Binding of Gcs to the GR stimulates phosphatidylinositol 3-kinase (PI3K) and the protein kinase AKT (Beck et al. 2009) in some cells. These effects result in rapid changes in the cytoplasmic environment that can lead to endothelial nitric oxide synthase (eNOS) activation and nitric oxide-dependent vasorelaxation (Hafezi-Moghadam et al. 2002) amongst other effects. Some of the immunosuppressive effects of Gcs are mediated by non-genomic signalling. In T cells, unliganded GR is associated in a complex with the antigen activated T cell receptor (TCR) and is necessary for TCR signalling. Gcs cause dissociation of the complex and result in TCR signalling blockade (Löwenberg et al. 2006).

GR is ubiquitously expressed although there are differences between tissues and the effect of Gcs in different tissues is influenced by a combination of the expression of different splice variants and co-regulator proteins and the role of tissue-dependent post-translational modifications. This is exemplified by high expression levels of the GR-C3 isoform in bone cells that is associated with high levels of Gc-induced apoptosis (Lu et al. 2007).

**Mechanisms of apoptosis relevant to Gcs**

Programmed cell death, also referred to as apoptosis, is carried out by a family of proteins known as caspases (Fig. 2). Effector caspases are the final target in the signalling pathway referred to as the ‘caspase cascade’. When effector caspases become active, the cell will undergo apoptosis since their role includes mediating nuclear breakdown and cellular degradation. Effector caspases are activated by inducer caspases, which are tightly regulated by a complex network of signalling proteins.

There are two pathways which trigger apoptosis: the extrinsic pathway is dependent on ligand binding and subsequent activation of membrane-bound ‘death signal’ receptors. Activating these receptors triggers the caspase cascade, resulting in apoptosis (Elmore 2007). The second pathway is the more complex intrinsic pathway (Elmore 2007). This pathway is regulated by members of the Bcl-2 family of proteins, which play a crucial role in the regulation of apoptosis.

**Figure 2** Mechanisms of glucocorticoid-mediated apoptosis. Glucocorticoids can exert their effects either genomically or non-genomically. While the exact pathway is not clear and may vary in different cell types it is thought that glucocorticoids act via the mitochondrial pathway to cause caspase activation.
family consisting of pro- and anti-apoptotic proteins. Members of the Bcl-2 family control many forms of apoptotic cell death by ‘tipping the balance’ from anti- to pro-apoptotic Bcl-2 family members. This balance is termed the ‘bcl-2 rheostat’ (Ploner et al. 2008). Cellular stress, such as DNA damage, leads to an increase in pro-apoptotic protein levels which in turn allows pro-apoptotic Bcl-2 proteins to induce the release of cytochrome c from mitochondria, causing activation of the caspase cascade (Adams & Cory 1998).

Effects of Gcs on apoptosis

The mechanism behind Gc-induced apoptosis is not fully understood and seems to vary depending on cell type. The levels of GR expression are important as evidenced by work in transgenic mice with increased (Reichardt et al. 2000) and decreased GR expression (Pazirandeh et al. 2002). Gcs can prevent activated upregulation of FasL but not Fas signalling as such, demonstrating that GR interferes with FasL expression in T cell hybridomas (D’Adamio et al. 1997, Ashwell et al. 2000). This indicates that Gcs can act on the extrinsic pathway.

There is considerable evidence that in almost all cell types studied, Gcs act via the intrinsic pathway. Gcs can activate cell death through induction of pro-apoptotic members of the Bcl-2 family, such as Bim, Bid and Bad (Han et al. 2001, Wang et al. 2003, Lu et al. 2007) and/or repression of anti-apoptotic members, such as Bcl-2, Mcl-1 and Bcl-xL, (Rogatsky et al. 1999, Casale et al. 2003, Chauhan et al. 2003, Lu et al. 2007). Gc-induced apoptosis is also compromised in thymocytes from mice deficient in pro-apoptotic proteins Apaf1 (Yoshida et al. 1998) or caspase 9 (Kuida et al. 1998). In addition, thymocytes from double knockout mice lacking Bak and Bax are resistant to Gc-induced apoptosis (Rathmell et al. 2002).

In addition to the well-described nuclear mechanisms of Gc action, steroid hormones have also been shown to directly or indirectly affect mitochondrial function (Manoli et al. 2007). In several cell lines, GR translocation to the mitochondria was observed in correlation with susceptibility to Gc-induced apoptosis via the ligand-induced mitochondrial pathway rather than nuclear translocation of the GR (Sionov et al. 2006a,b). However, mitochondrial translocation of GR was only observed in cells responsive to Gc-induced apoptosis, and not in Gc-resistant types. This presents a qualitative difference in GR behaviour that may explain the differential response of some cancer cells to Gc therapy.

Gc-induced apoptosis in tumour cells and mechanisms of resistance

Most of the data obtained so far on the mechanisms of Gc-induced apoptosis in tumour cells is from work done in acute lymphoblastic leukaemia (ALL), osteosarcoma and small-cell lung cancer. In all of these tumour types there are examples of resistance to Gc actions. In fact, most cancer cells derived from malignant solid tumours seem to exhibit resistance towards Gc-induced apoptosis, which makes the comparison between different tumour types even more important (Herr et al. 2009).

Resistance to Gc-induced apoptosis in lymphoblastic leukaemia

There are extensive studies outlining the mechanisms by which Gcs cause cell death in ALL. It is well known that Gcs induce apoptosis in some lymphoid cells, such as immature thymocytes and ALL cells (Smith & Cidlowski 2010) and this has subsequently led to treating lymphoid malignancies with Gcs (Gaynon & Carrel 1999). However, resistance to Gcs has been identified as a significant feature during treatment and insight is needed on how resistance develops in these tumour cells to improve Gc-mediated therapies in haematological malignancies.

The mechanisms by which Gcs induce apoptosis in ALL are not clearly defined and this makes understanding of acquired resistance difficult. It is known that Gc-mediated apoptosis in ALL requires the interaction of the Gc ligand with its receptor. This is exemplified by failure of apoptotic induction in human ALL cell lines expressing mutant forms of GR (Hala et al. 1996). However, Gc sensitivity in resistant ALL cell lines can be restored by introducing exogenous wild-type GR (Helmberg et al. 1995, Geley et al. 1996). Further studies in human T-ALL cell lines expressing differing levels of GR showed that GR expression above a certain threshold is vital for Gc sensitivity (Geley et al. 1996) and similar observations were later made in Gc-sensitive and Gc-resistant multiple myeloma lines (Chauhan et al. 2002). Interestingly, washing out Gcs before 24 h prevented ALL cells from undergoing apoptosis (Brunet et al. 1998). This suggests that GR levels above a certain threshold and appropriate ligand availability are required for some time to cause apoptosis triggering in ALL cells.

Gc-triggered apoptosis is believed to occur via changes in gene expression but discerning if this is due to transactivation, transrepression or a combination of the two proves to be difficult. Gc-resistant Jurkat cells and CEM cells were sensitised to Gcs by expressing transactivation-deficient GR mutants constitutively, leading to the conclusion that transrepression alone was sufficient for apoptosis (Thompson et al. 1992, Helmberg et al. 1995). However, the opposite result was presented from work done in mouse thymoma cells deficient for GR, which were transfected with N-terminal-deleted GR constructs (Dieken & Miesfeld 1992). In this instance transactivation was at least partially required as cells were only able to undergo apoptosis once a VP-16 transactivation domain had been transfected in. VP-16 is thus acting as a substitute for the missing GR transactivation domain. Based on these contradictory findings it appears that upon Gc binding to GR there are multiple mechanisms of altering gene expression in favour of pro-apoptotic signals.
The likelihood that instead of a single pathway causing Gc-induced apoptosis in ALL there are multiple mechanisms, depending on cell origin and tissue environment, poses a problem when it comes to pinpointing target genes involved in triggering Gc-mediated apoptosis. Gcs may activate apoptosis through direct regulation of components of either the extrinsic or intrinsic apoptotic pathway. Inhibition of caspase 8 in human ALL cell lines did not prevent the cells from undergoing apoptosis (Geley et al. 1997, Planey et al. 2003) and this may indicate that the extrinsic pathway is not necessary for Gc-mediated apoptosis. Studies in human ALL cell lines showed that members of the anti-apoptotic Bcl-2 protein family were able to diminish Gc-mediated apoptosis in human ALL cell lines when overexpressed (Brunet et al. 1998, Hartmann et al. 1999). It has become evident that Gc-mediated apoptosis includes the upregulation of pro-apoptotic (Han et al. 2001, Wang et al. 2003) and/or downregulation of anti-apoptotic genes (Chauhan et al. 2002, Casale et al. 2003) and this would therefore suggest that altering the expression levels of proteins involved in the Bcl-2 rheostat provides a mechanism for explaining Gc-mediated apoptosis (Schmidt et al. 2004).

In some cases, it is possible that the GR can cause apoptosis through non-genomic mechanisms, for example by the rapid activation of protein kinases such as MAPK, PI3K and AKT, which in turn activates eNOS (Limbourg & Liao 2003).

**Mechanisms of Gc resistance in lymphoid malignancies**

The necessity for prolonged treatment with Gcs in ALL makes it prone to development of Gc resistance. In addition, those patients who relapse have increased Gc resistance (Schrapp et al. 2000) and this resistance is associated with poor prognosis. In various malignant cell lines resistance is caused by mutations in the GR (Ashraf & Thompson 1993, Powers et al. 1993, Strasser-Wozak et al. 1995, Hala et al. 1996, Rimil et al. 2004, Schmidt et al. 2006). However, there are only a few examples of GR mutations causing a resistant phenotype in primary cells from cancer patients displaying Gc resistance. To date, the only known acquired GR mutations associated with Gc resistance in *in vivo* malignancies are the L753F and Δ702 mutants in the ligand-binding domain of the GR. L753F was discovered in cells from a patient with Gc-resistant ALL with the mutant being transactivation deficient (Hillmann et al. 2000). The Δ702 mutant was also discovered in cells from a patient with Gc-resistant ALL and this mutant has a decreased affinity for ligand binding (Irving et al. 2005).

Resistance to Gcs can occur by downregulation of the GR following Gc treatment (Kfir et al. 2007). Decreased expression of the GR may occur through decreased activity of the GR promoter, decreased mRNA stability (Schaaf & Cidlowski 2002), or decreased protein stability (Wallace & Cidlowski 2001). Furthermore, there is evidence of alterations in the expression levels of GR isoforms, which might contribute towards Gc resistance. GRβ expression is increased in CEM cells and Gc-resistant CLL (Haarman et al. 2004), although there was no correlation between Gc resistance and GRβ expression in primary cells from patients with ALL (Haarman et al. 2004) where there was a correlation with GRγ expression. There is also increased GR-P expression in Gc-resistant tumour cells but not primary ALL cells (Tissing et al. 2005).

Given the plethora of potential changes in the GR in resistant ALL cells it may be necessary to focus on targets further downstream, such as modulation of Bim expression, to increase apoptosis. However, yet again these pathways seem very diverse and whether a single mechanism will overcome the resistance remains controversial (Smith & Cidlowski 2010).

**Resistance to Gc-induced apoptosis in osteosarcoma cells**

It has been suggested that Gc resistance is a hallmark of osteosarcoma development due to the fact that Gcs cause apoptosis in osteoblast cells (Weinstein et al. 1998). In an animal model where 11-β-hydroxysteroid dehydrogenase 2, an enzyme that reduces corticosteroid levels, is overexpressed, it appears that Gcs exert their apoptotic effect directly on bone cells (O’Brien et al. 2004). The drive to remove GR from the cell in the human osteosarcoma cell line, U2OS, negates any Gc-induced effects on inhibition of proliferation via G1 cycle arrest (Rogatsky et al. 1997, 2003, Takayama et al. 2006). The pathway of the apoptosis trigger in U2OS cells is however still largely unknown. There is evidence that decreased Bcl-2 may be responsible for Gc-mediated apoptosis (Rogatsky et al. 1999) and downregulation of anti-apoptotic factors such as Bcl-xL and Mcl-1 has been observed in U2OS cells undergoing apoptosis (Lu et al. 2007). Another complexity in Gc-mediated apoptosis in osteosarcoma cells occurs due to specific effects of different GR isoforms produced via alternative translation initiation mechanisms, which result in varying lengths of the N-terminus of GR. (Lu & Cidlowski 2005). The GR–C isoform was more active in regulating genes involved in apoptosis that may be due to selective recruitment of co-factors (Lu et al. 2007), while expression of the GR–D isoform reduced Gc-mediated cell death suggesting that expression of this isoform may lead to Gc resistance.

**Resistance to Gc-induced apoptosis in small-cell lung cancer cells**

Some patients with small-cell lung cancer (SCLC) develop the ectopic ACTH syndrome because their tumours secrete the hormone ACTH (or in most cases, its prohormone, proopiomelanocortin (POMC)). In the pituitary, expression of the POMC prohormone and ACTH secretion is inhibited by Gcs in a negative feedback loop. However, SCLC tumours
are resistant to Gc inhibition and this has long been used as the basis of one of the main diagnostic tests for this syndrome (Oliver et al. 2003).

This led to the study of Gc resistance in a panel of SCLC cell lines that secrete POMC (Stewart et al. 1989, White et al. 1989, Clark et al. 1990). A common theme that became apparent was that the functionality of the GR was impaired. This occurred in one cell line because of a mutation in the GR gene resulting in the truncated GR form known as GRd (also known as GR–P) being expressed (Gaitan et al. 1995, Turney & Kovacs 2001). In another cell line, one allele of the GR had an amino acid transition in the zinc finger domain of the DNA-binding region of the GR, which resulted in GRγ (Ray et al. 1994, 1996). In addition, the overexpression of the GR co-repressor NCOR leads to reduced transactivation and is therefore believed to be responsible for Gc resistance in the SCLC cell line, CORL 103 (Waters et al. 2004). This is in line with other observations that point towards abnormal co-regulator expression as a mechanism in cancers and metabolic disease including Gc resistance (Lonard et al. 2007).

Recently, we have identified an overall decrease in GR expression across a panel of SCLC cell lines (Somer et al. 2007 and unpublished data).

As the SCLC cell lines possess reduced GR functionality, the effects of restoring expression were investigated. Transient transfection of wild-type GR restores Gc sensitivity in the SCLC cell line, DMS 79 (Ray et al. 1994, Sommer et al. 2007). Treatment with Gcs had an inhibitory effect on proliferation in a non-SCLC cell line, but not in DMS 79 cells. When an exogenous form of the GR was overexpressed in DMS 79 cells via retroviral infection, a significant increase in cell death was observed, which was inhibited with the GR antagonist RU486. Transient overexpression of GR in DMS 79 cells resulted in >90% loss in live cells within 72 h. Microarray analysis demonstrated that GR overexpression caused an increase in expression levels of the pro-apoptotic genes, Bad and Bax (Sommer et al. 2007).

This pro-apoptotic effect of GR was substantiated in vivo in SCLC xenografts that were infected with a GR–expressing adenovirus that caused significantly decreased tumour growth (Sommer et al. 2010). A significant increase in TUNEL-positive cells was observed. Interestingly, a number of uninfected cells were also shown to be apoptotic, suggesting a bystander effect on surrounding cells. The GR-induced apoptosis in DMS 79 xenografts appeared to involve decreased expression of the anti-apoptotic genes Bcl-2, Bcl-xL and Mcl-1 (Sommer et al. 2010).

Low level expression of GR in the panel of SCLC cell lines, which is associated with increased promoter methylation (unpublished data), is similar to mechanisms associated with conventional tumour suppressor genes. Understanding how restoration of GR expression results in tumour cell death may uncover new therapeutic strategies in SCLC treatment in the future.

Summary

The pleomorphic effects of Gcs are underpinned by a wide range of mechanisms that mediate Gc actions in different cell types. Not surprisingly, this has led to multiple mechanisms being implicated in Gc resistance making it difficult to identify ways to overcome Gc resistance in patients. This is particularly important as Gcs are one of the most effective agents for lymphoid malignancies and are used to treat many cancers not only as pro-apoptotic agents but also to reduce emesis associated with chemotherapy. There is a possibility that treatment with Gcs induces the resistant phenotype in cells of solid tumours while having pro-apoptotic properties in lymphoid malignancies. This would imply that Gcs may be crucial in causing faster growth and metastasis of solid tumours by providing a selection pressure. However, since there are examples of Gc resistance in lymphoid tumour cell lines it may be that solid tumours become resistant more easily due to their growth characteristics, whereas lymphoid tumours take longer to develop resistance because of their morphology. If we are to find therapeutic approaches to combat Gc resistance we will need a greater understanding of key checkpoints in these Gc-regulated pathways.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

G S is funded by the BBSRC and a Barbara Mawer Trust studentship.

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Received in final form 16 May 2011
Accepted 20 May 2011
Made available online as an Accepted Preprint 20 May 2011