Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease

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Received: September 10, 2010  Revised: December 21, 2010
Accepted: December 28, 2010
Published online: March 7, 2011

Abstract

Natural and synthetic glucocorticoids (GCs) are widely employed in a number of inflammatory, autoimmune and neoplastic diseases, and, despite the introduction of novel therapies, remain the first-line treatment for inducing remission in moderate to severe active Crohn’s disease and ulcerative colitis. Despite their extensive therapeutic use and the proven effectiveness, considerable clinical evidence of wide inter-individual differences in GC efficacy among patients has been reported, in particular when these agents are used in inflammatory diseases. In recent years, a detailed knowledge of the GC mechanism of action and of the genetic variants affecting GC activity at the molecular level has arisen from several studies. GCs interact with their cytoplasmic receptor, and are able to repress inflammatory gene expression through several distinct mechanisms. The glucocorticoid receptor (GR) is therefore crucial for the effects of these agents: mutations in the GR gene (NR3C1, nuclear receptor subfamily 3, group C, member 1) are the primary cause of a rare, inherited form of GC resistance; in addition, several polymorphisms of this gene have been described and associated with GC response and toxicity.

However, the GR is not self-standing in the cell and the receptor-mediated functions are the result of a complex interplay of GR and many other cellular partners. The latter comprise several chaperonins of the large cooperative hetero-oligomeric complex that binds the hormone-free GR in the cytosol, and several factors involved in the transcriptional machinery and chromatin remodeling, that are critical for the hormonal control of target genes transcription in the nucleus. Furthermore, variants in the principal effectors of GCs (e.g. cytokines and their regulators) have also to be taken into account for a comprehensive evaluation of the variability in GC response. Polymorphisms in genes involved in the transport and/or metabolism of these hormones have also been suggested as other possible candidates of interest that could play a role in the observed inter-individual differences in efficacy and toxicity. The best-characterized example is the drug efflux pump P-glycoprotein, a membrane transporter that extrudes GCs from cells, thereby lowering their intracellular concentration. This protein is encoded by the ABCB1/MDR1 gene; this gene presents different known polymorphic sites that can influence its expression and function. This editorial reviews the current knowledge on this topic and underlines the role of genetics in predicting GC clinical response. The ambitious goal of pharmacogenomic studies is to adapt therapies to a patient’s specific genetic background, thus improving on efficacy and safety rates.

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Key words: Glucocorticoids; Inflammatory bowel disease; Pharmacogenomics

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De Iudicibus S, Franca R, Martelossi S, Ventura A, Decorti G.
Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease. World J Gastroenterol 2011; 17(9): 1095-1108
Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i9/1095.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i9.1095
INTRODUCTION

Glucocorticoids (GCs) are a well-accepted therapy for inflammatory, autoimmune and proliferative diseases. Despite the large clinical use, the benefits of these agents are often narrowed by inter-individual variability. Indeed, some patients show a poor or absent response, and in these subjects, GCs have to be employed in high doses. The clinical use of these compounds is associated with a number of serious complications, including osteoporosis, metabolic disease and increased risk of cardiovascular disease; therefore, patients, and in particular those who respond poorly to these agents, are at high risk of side effects. In addition, besides being a problem for patients and a challenge to clinicians, inadequate GC therapy also represents a socio-economic matter because of the consequent considerable impact on health care costs.

GCs are effective inhibitors of cytokine secretion and T-cell activation, and are consequently largely employed in different inflammatory conditions, including inflammatory bowel disease (IBD). In these diseases, GC resistance or dependence is particularly frequent. Clinical reports in pediatric IBD patients have shown that up to 90% of subjects have a rapid improvement of symptoms when prednisone is given. However, after 1 year, only 55% of early steroid-treated patients are still in remission and can be considered steroid-responsive. Around 38% of patients are not able to discontinue the therapy and experience an increase in disease activity when the dose is reduced (steroid-dependent); 7% of subjects are resistant and do not respond to GC therapy. Among the adult IBD population, a prospective analysis has described the 1-year outcome in patients with Crohn’s disease (CD) treated with a first oral prednisolone course (40-60 mg/d) and tapering to a maintenance dose of 10-15 mg/d. In this study, prolonged steroid response was obtained in 44% of patients, 36% of subjects were steroid dependent and 20% steroid resistant, and a high frequency of surgery was reported within 1 mo after steroid treatment. Similar results have been obtained in a retrospective American study: immediate outcomes for CD and ulcerative colitis (UC) respectively, were complete remission in 58% and 54%, partial remission in 26% and 30%, resistance in 16% of patients. Outcome at 1 year showed a prolonged response in 32% of CD patients, GC dependence in 28%, and surgical intervention in 38%. In UC patients, 1-year outcomes were prolonged response in 49%, dependence in 22%, and surgery in 29% of subjects.

In 1999, a pivotal study by Hearing et al. demonstrated a poor in vitro response to GCs of circulating lymphocytes in UC patients who exhibited a reduced or absent clinical response to these agents. In vitro lymphocyte resistance to GCs has been demonstrated to correlate with clinical outcome in other inflammatory diseases such as asthma, systemic lupus erythematosus, rheumatoid arthritis and renal allograft rejection. A wide variation in lymphocyte steroid sensitivity is evident even in healthy individuals. This suggests that the observed variability is a stable intrinsic property of an individual that becomes important when the subject has to be treated with GCs for an inflammatory disease, and is therefore likely to have a genetic basis.

Over the past years, significant advances have been made in understanding the molecular mechanisms by which GCs act and the basis of such inter-patient variability. This editorial describes the mechanisms of GC anti-inflammatory action and discusses the molecular and genetic basis for GC resistance in chronic inflammatory diseases, especially IBD.

MOLECULAR MECHANISM OF GC ACTION

Due to their high lipophilicity, exogenous GCs are widely bioavailable; these agents, as well as the endogenous compound cortisol, are transported in the blood predominantly bound to the corticosteroid-binding globulin and, to a lesser extent, to albumin. Free GCs are able to diffuse passively across plasma membranes and interact specifically with a cytosolic receptor (GR), expressed in virtually all tissues. The GC receptor is a member of the nuclear receptor (NR) superfamily, which includes receptors for steroid hormones (e.g. corticosteroids, androgens, estrogens and progesterone), as well as other hydrophobic molecules (such as bile acids, vitamins A and D, retinoic acid and thyroid hormones). All these molecules induce their actions by the same molecular mechanism: at the basis of this mechanism stands the physical interaction between the lipophilic ligand and its own cytosolic/nuclear receptor that, in turn, activates a multistep signal transduction pathway to end up in specific genomic transcriptional effects. Receptors belonging to the NR superfamily are highly homologous to each other and share structural features with a common modular domain organization: they present a transactivation domain at the N-terminal part (NTD), a central zinc finger DNA-binding domain (DBD) and a ligand-specific binding domain (LBD) at the C terminus.

The ligand-free GR is not self-standing in the cell, but exists in heteromeric complex with molecular chaperones and co-chaperones. In the functionally mature form of the heterocomplex, the free GR is associated with an Hsp90 dimer, with p23 and with any of the Hsp90 co-chaperones, except Hsp70/Hsp90 organizing protein. Such associations are essential for keeping the receptor in the correct folding for a hormone-responsive state. Upon ligand binding, receptors undergo conformational changes and expose the DBD, which is otherwise hidden in the ligand-free conformation. Cytosolic receptors also provide nuclear localization signals (NLSs) that are comprised of a closely spaced arrangement of 5-8 basic amino acids, which co-localize with DBD and, once exposed, interact with nuclear transport factors (importins).

Nuclear heterocomplex translocation is necessary for transactivating target genes. Translocation is a tightly regulated process that occurs through nuclear pore complexes in a ligand- and energy-dependent manner, and is mediated by specific nuclear transport factors that belong to the evolutionarily conserved importin β family of nuclear transporters. Among these, importin-13 (IPO13) has
been functionally characterized as a primary regulator of the translocation of the GC-bound GR across the nuclear membrane. The zinc finger motifs of the DBD allow the interaction of the activated receptor with specific DNA sequences, termed GC-responsive elements (GRE, 5′-GGTA-
CAANNTTGTTTCT-3′ where n refers to any nucleotide), located within regulatory regions of GC-responsive genes. GR homodimerizes on GRE and recruits transcriptional co-activators, as well as basal transcription machinery, to the translocation start site. These co-activators, that include CREB (cAMP response element-binding) binding protein (CBP), steroid receptor co-activator-1 (SRC-1), GR-interacting protein (GRIP-1), p300 and switching/sucrose non-fermenting chromatin remodeling complex (SWI/SNF), induce histone acetylation, thus allowing the transactivation of GC-responsive genes. Although some GC anti-inflammatory effects are achieved through induction of anti-inflammatory genes, such as interleukin (IL)-10, annexin 1 and the inhibitor of nuclear factor (NF)-κB, transactivation enhances mainly the expression of genes involved in metabolic processes, and is therefore, responsible for the majority of unwanted side effects.

Although positive GREs mediate transcriptional up-regulation in response to GCs, negative GREs (5′-ATY-
ACNNTrTGATCn-3′) downregulate the transcription of responsive genes. Indeed, the presence of GR on GRE might competitively prevent the binding of activator protein (AP)-1 and NF-κB on the same promoter regions or might transactivate their inhibitor proteins. Furthermore, GRE-independent mechanisms of transrepression also exist: the GR physically interacts with AP-1 [30, 31], NF-κB [32] and signal transducers and activators of transcription [33]. Transrepression is believed to be responsible for the majority of the beneficial anti-inflammatory effects of GCs [30, 31, 32, 33, 34].

The molecular mechanisms of GC action are further complicated by the utilization that these hormones can also induce rapid non-genomic effects within the cytoplasm; for example, they induce the release of Src kinase from the GR heterocomplex, which results in lipidocortin activation and inhibition of arachidonic acid release, and altered cytoplasmic ion content.

**MOLECULAR MECHANISM OF GC RESISTANCE IN IBD**

The phenomenon of GC resistance in chronic inflammatory diseases is, as stated above, quite common, however the precise molecular mechanism is still unclear. First of all, it should be separated from the rare familial condition of primary generalized GC resistance, for which the name of Chrousos syndrome has been recently proposed. This is a rare, sporadic or familiar syndrome caused by mutations in the nuclear receptor subfamily 3, group C, member 1 (NR3C1, Nuclear Receptor Nomenclature Committee, 1999) gene. The disease is characterized by target tissue insensitivity to GCs due to reduction or lack of functional GC receptors and by compensatory elevation in adrenocorticotropic hormone (ACTH). This results in an increased secretion of cortisol, albeit in the absence of signs of Cushing syndrome, as well as of other adrenal hormones with mineralocorticoid and androgenic activities, which is responsible for the main symptoms (hypertension and signs of hyperandrogenism). As stated above, this syndrome is, however, extremely rare, and no cases in IBD patients have been described in the literature.

In consideration of the complexity of GC mechanisms of action (Figure 1), the most common forms of resistance observed in chronic inflammatory conditions, and in IBD in particular, may occur at several levels, and some candidate areas have been identified (Tables 1 and 2): (1) the GR receptor heterocomplex and proteins involved in nuclear translocation and transcription; (2) the pro-inflammatory mediators in the downstream signaling pathway of the GC-GR complex; and (3) P-glycoprotein (P-gp) and other proteins involved in the extrusion and metabolism of GCs.

**The GR heterocomplex and proteins involved in nuclear translocation and transcription**

The GR is an interesting candidate for GC resistance in IBD, and a significant lower expression of GR mRNA has been reported in the intestinal mucosa in patients with steroid-resistant UC; in addition, GC binding affinity in mononuclear cells is reduced in patients with GC resistant diseases.

| **Table 1** | Factors involved in glucocorticoid resistance |
|-------------|---------------------------------------------|
| **GR**      | Reduced GR mRNA expression [30]             |
|             | Reduced GC binding affinity [44]            |
| **GR heterocomplex** | Altered expression of the chaperones and co-chaperones that make up the heterocomplex [24, 36] |
|             | Polymorphisms in the Hsp90 gene [29]        |
| **Nuclear transport factors** | Genetic variations in the STIP1 gene coding for Hop [29] |
| **Transcription machinery** | Lower expression of SMARC1 in resistant leukaemia cells [37] |
| **Pro-inflammatory mediators** | Polymorphisms in the NALPi gene [32] |
| **P-gp**    | TNF-α promoter SNP correlated with steroid response in several disease [40-44] |
|             | IL-10 polymorphisms related with a positive prednisone response in children with ALL [30, 34] |
|             | Hyperexpression of P-gp in lymphocytes and epithelial cells from GC-resistant IBD patients [20, 27] |

**GR:** Glucocorticoid receptor; **GC:** Glucocorticoid; **Hsp:** Heat-shock protein; **Hop:** Hsp70/Hsp90 organization protein; **P-gp:** P-glycoprotein; **NR3C1:** Nuclear receptor subfamily 3, group C, member 1; **NALPi:** NACHT leucine-rich-repeat protein 1; **SNP:** Single nucleotide polymorphism; **TNF-α:** Tumor necrosis factor α; **IL:** Interleukin; **ALL:** Acute lymphoblastic leukemia; **IBD:** Inflammatory bowel disease.
The human GR gene NR3C1 is located on chromosome 5q31.3 and includes nine exons. Polymorphisms, that is, variations in the DNA sequence with a frequency of more than 1% in the healthy population, of the human GR gene may impair the formation of the GC-GR complex and subsequently alter transactivation and/or transrepression processes.

A large number of polymorphisms in this gene have been described: according to the dbSNP build 130 database of National Center for Biotechnology Information (NCBI), 1152 entries are known at the moment, but only a few are functionally relevant. The TthIII I (rs10052957), ER22/23EK (rs6189/rs6190), N363S (rs6195), BclI (rs41423247) and GR-9β (rs6198) polymorphisms have been the most studied and have been associated with differences in metabolic parameters and body composition, and with autoimmune and cardiovascular disease. These genetic variants have been also related with changes in GC sensitivity or altered cortisol level and may therefore account for the variability in the response to GC therapy.

Three polymorphisms are associated with a reduced sensitivity to endogenous and exogenous GCs. TthIII I is a restriction fragment length polymorphism (RFLP) caused by a C>T change in the GR gene promoter region; it is located in a large intron of approximately 27 kb, 3807 bp upstream of the GR start site. The polymorphism has been associated with elevated diurnal cortisol levels and with a reduced cortisol response to 1 mg dexamethasone (DEX), as well as lower insulin and cholesterol levels.

The ER22/23EK polymorphisms are located in the N-terminal transactivation domain of the GR and involve...
two nucleotide changes in codon 22 and 23 of exon 2 (GAG AGG to GAA AAG), which are changing the amino acid sequence from glutamic acid-arginine (E-R) to glutamic acid-lysin (E-K). Since the polymorphism is located in the transactivation domain, the amino acid change might affect the tertiary structure of the receptor, which influences the transactivational and/or transrepressional activity on target genes. A relative GC resistance, with a reduction of GR transcriptional activity in transfected COS-1 cells and in peripheral blood mononuclear leukocytes of homozygous carriers, has been described. In vivo, an association with higher post-DEX cortisol levels as well as less cortisol suppression after a 1 mg DEX suppression test in ER22/23EK carriers, has also been shown. In addition, the polymorphism is associated with a better metabolic and cardiovascular health profile and an increased survival.

The GR-9β polymorphism is located in the 3’-untranslated region (UTR) of exon 9β, where an ATTTA sequence found between the presence of this SNP and response to exogenous GCs. Szabó et al. have investigated whether variants of the NR3C1 gene may contribute to steroid-induced ocular hypertension. In 102 patients who underwent photorefractive keratectomy and received topical steroids as part of postoperative therapy, a significant correlation was observed between N363S heterozygosity and ocular hypertension. Furthermore, in 48 patients with Duchenne muscular dystrophy treated with prednisolone or deflazacort, the N363S carriers showed a trend towards a later age at loss of ambulation in comparison with non-carrier patients. Only one study so far has evaluated the role of this polymorphism in GR response in IBD: but no relation was observed between the presence of this SNP and response to GCs in a population of 119 pediatric patients.

As extensively reported in the literature, the most clinically relevant polymorphism of the NR3C1 gene is the Bcl I SNP. Initially described as a polymorphic restriction site inside intron 2, the nucleotide alteration was subsequently identified as a C>G substitution, 646 nucleotides downstream from exon 2. This polymorphism is also associated with hypersensitivity to GCs in both heterozygous and homozygous carriers of the G allele. An association with unfavorable metabolic characteristics, such as increased body mass index and insulin resistance has been also described.

The Bcl I SNP has been studied in 119 pediatric patients with IBD (64 with CD, and 55 with UC). Patients were divided into two groups based on their response to GC treatment: GC dependence (45 patients) was defined by an initial response to prednisone with relapse on dose reduction, not allowing steroid discontinuation, and GC-responsiveness (67 patients), equivalent to therapeutic success, was defined as GC withdrawal without the need for steroids for at least 1 year. A significantly higher frequency of BclI mutated genotype was observed in the GC-responsive patients than in the GC-dependent group, which confirms an increased sensitivity to GCs in subjects with the BclI mutated genotype. These results have been subsequently confirmed in a larger cohort of young patients with IBD.

**GR heterocomplex:** The integrity of the mature GR heterocomplex is required for optimal ligand binding and subsequent activation of the transcriptional response, and abnormalities in the chaperones and co-chaperones that make up the heterocomplex may contribute to altered GC responsiveness.

Although no study has considered the role of these abnormalities in IBD, altered levels of Hsp90 have been...
found in peripheral blood mononuclear cells from individuals with steroid-resistant forms of asthma[84] and idiopathic nephrotic syndrome[85]. Kojika et al[86] have shown that alteration in Hsp90 and Hsp70 is associated with decreased sensitivity to GCs in human leukemia cells. However, no relationship has been found between Hsp90 mRNA and resistance to GCs in spare bone marrow or peripheral blood samples taken from ALL patients at diagnosis[87]. SNPs in the Hsp90 genes (HSPCA encoding hsp90-1α, HSPCB encoding hsp90-1β) have been recently described; however, in an adult asthmatic population, no correlation was found between SNPs in the HSPCA (3′-UTR+307, rs3736807, rs4906178, rs3809386, promoter -32) or HSPCB (rs504697 and rs3757286) genes and response to treatment[88].

Generally, steroid receptors display functional instability when deprived of chaperones. FKBP51, coded by the FKBP5 gene, is a negative regulator of GC action and reduces GR binding affinity[89]. However, when GCs bind to the GR, the receptor complex is activated and FKBP51 is replaced by FKBP52 (coded by FKBP4 gene), a positive regulator of GR signaling[90]. Among the relative levels of FKBP51 and FKBP52 have been shown to be important determinants of GC cellular sensitivity in various systems. One recent study has investigated the role of FKBP5 genetic variants in the response to GCs in asthmatic patients[97], however the studied polymorphisms (rs3800373, rs9394309, rs938525, rs9470080, rs9368878 and rs3798346) were not correlated with response. In this study, genetic variations in the STIP1 gene, which codes for the co-chaperone Hsp70/Hsp90 organizing protein, on the contrary, seemed to have a role in identifying asthmatic subjects who were more responsive to GC therapy[90].

Nuclear transport factors: Importins represent important players in GC pharmacology, as they mediate nuclear translocation through nuclear pore complexes. Importin 13 (IPO13) has been functionally characterized as a primary regulator of GC-bound GR across the nuclear membrane[80]. IPO13 was first discovered as a GC-inducible gene that is important in lung development[83], therefore, its functional characterization has been performed in airway epithelial cells and lung-derived transformed cell lines[90]. Inhibition of lung epithelial cell IPO13 production reduces the nuclear translocation of GR from the cytoplasm, and subsequent GC-mediated silencing of inflammatory cytokine production[84], which suggests that the normal anti-inflammatory response induced by GCs is dependent on normal IPO13 expression levels or activity. Therefore, irregular GC-GR signaling, ascribable to IPO13 variation, might affect the therapeutic responsiveness to GCs. IPO13 is expressed in other tissues as well, such as fetal brain, heart, kidneys and intestine[83,85], therefore, it is feasible to hypothesize that dysregulation of IPO13-mediated processes might account for the variable response to GCs also in other diseases, such as IBD.

Importins are interesting candidates for pharmacogenomic studies, although little is known about their genetic variants. Recently, the association of 10 IPO13 polymorphisms with responsiveness to inhaled GCs, measured by change in methacholine dose required for a 20% drop in FEV1, has been investigated in children with mild to moderate asthma[86]. Unexpectedly, the genetic effects conferred by IPO13 variants were clinically significant in subjects who were randomized to placebo and nedocromil treatment (control group), rather than those treated with the inhaled GC budenoside, either in single SNP or in haplotype analysis. No other study on IPO13 genetic variants is available to date, therefore, these results require confirmation and further investigations are needed to shed light on the putative role of IPO13 mutants in GC-resistant or GC-hyper-responsive cases in other chronic inflammatory diseases.

Transcription machinery: In eukaryotic cells, gene transcription is inhibited by condensed chromatin structure, which prevents the interaction between gene-specific transcription factors and their DNA recognition sequences, thus blocking the access of the transcriptional machinery to DNA[90]. Dynamic chromatin remodeling is therefore a fundamental mechanism in mediating genomic effects, and a large family of protein complexes that promote chromatin restructuring in an ATP-dependent manner, by disrupting histone-DNA contacts, has been described[91-93]. Among these, the highly conserved mammalian SWI/SNF chromatin remodeling complex is the one recruited during GC-dependent gene activation[91-93]. The complex is composed of several subunits[94,95], including a catalytic ATPase subunit (either SMARCA4/BRG1 or SMARCA2/BRM) and Brahma-related gene 1 (BRG1)-associated proteins[96], such as the SWI/SNF-related, matrix-associated, actin-dependent regulators of chromatin (SMARC)C1, SMARCC2, SMARCD1-3, SMARCE1, SMARCB1 and ACTL6-A-B[94,95].

Alterations in any component of the SWI/SNF complex might be responsible for GC resistance, if access of GC-bound GR to DNA is compromised because of an impaired or suboptimal chromatin remodeling and nucleosome disruption. Using a genome-wide approach in patients with newly diagnosed childhood B-lineage ALL, differential expression of a relatively small number of genes has been shown to be associated with drug resistance and treatment outcome[91]. Lower expression of SMARCB1 is related to resistant leukemia cells and, vice versa, higher levels are associated with GC-sensitive ALL[77]. Decreased expression of other core subunits of the SWI/SNF complex, such as SMARCA4 and ARID1A, is also associated with GC resistance in primary ALL cells[98].

To date, only a few studies have addressed the functional effects of SMARC polymorphisms. By using the human CEPH (Centre d’Etude du Polymorphisme Humain) cell lines from 90 individuals and sequencing the SMARC1 promoter region, a regulatory SNP (-228G>T) has been discovered: the TT genotype has been functionally associated with higher SMARC1 expression at both mRNA and protein levels[94,95], and a positive association has been found between the SMARC1 mRNA level and prednisolone sensitivity, as evaluated by MTT assay. In addition, knocking down of SMARC1 in human ALL cell lines has confirmed that reduced SMARC1 expression induces GC resistance[99].
Pro-inflammatory mediators in the downstream signaling pathway of the GC-GR complex

Inflammation can be seen as a physiological homeostatic process elicited by the body in response to injurious agents, to protect tissues and to help in recovery. However, inflammation itself can potentially lead to tissue damage, if the organism does not properly control the process. Endogenous GCs are involved in the regulation of many physiological functions and assist the innate and adaptive immunity by balancing pro- and anti-inflammatory mediators, thus preventing overwhelming inflammation.

According to gene expression analysis of human peripheral blood mononuclear cells from healthy donors, approximately 20% of genes, particularly those involved in the immune response, are regulated either positively or negatively in response to DEX treatment. GCs downregulate the expression of pro-inflammatory cytokines [such as IL-1α, IL-1β, IL-8, interferon (IFN)-α and IFN-β], chemokines, adhesion molecules, inflammatory enzymes and receptors. At the same time, GCs upregulate the expression of other cytokines that suppress the production of inflammatory mediators (such as transforming growth factor-β and IL-10), thus boosting the anti-inflammatory effects.

Pro-inflammatory cytokines are involved in the pathogenesis of several chronic inflammatory diseases such as rheumatoid arthritis, osteoarthritis, asthma and IBD. Their excessive expression is generally efficiently counteracted by GCs. Individuals with steroid-non-responsive forms often show higher levels of local and/or systemic pro-inflammatory cytokines.

Research of the cytokine profile in relation to GC resistance has been mainly performed in steroid-resistant asthma, and only a few studies have involved patients with IBD. Several in vitro data suggest that cytokines modify GC effects by interference with the GR signaling. IL-1α has been reported to inhibit DEX-induced GR translocation in mouse fibroblasts and to downregulate the GR in a rat hepatoma cell line, whereas IL-1β inhibits GR function in colon epithelial cells. Tumor necrosis factor (TNF)-α decreases corticosenitivity in monocytes by downregulation of GR, and, in patients with UC, mucosal levels of this cytokine, as well as of IL-6 and IL-8 are higher in steroid-resistant patients. In a recent study, a large panel of cytokines was studied and their expression was correlated with steroid response. In particular, high IL-10 expression significantly enhanced steroid action, while IL-2 appeared to have the greatest antagonistic effect on the antiproliferative activity of steroids. This cytokine has also been related to increased GRβ expression, decreased translocation of the GR-GC complex to the nucleus, and increased AP-1 levels, with abnormal interaction with the GR. IL-2 and IL-4 reduce GR affinity and T-cell response to GCs in vitro, by a mechanism that involves p38 mitogen activated protein kinase (MAPK) activation. GCs inhibit the MAPK signaling pathway, through the induction of MAPK phosphatase 1 (MKP1), and this could result in inhibition of expression of various inflammatory genes in IBD.

Conclusion: a complex circular interplay between GCs and cytokines takes place, with GCs downregulating pro-inflammatory cytokines and cytokines limiting GC action. Basal cytokine expression levels are fine-tuned by genetic profile. Polymorphisms in the cytokine regulatory regions determine a “lower/higher cytokine producers” phenotype that might in part be responsible of inter-individual variations, in terms of severity of inflammation and therapy responses. Higher cytokine producers might become GC-resistant or they might be less responsive at standard doses, therefore requiring dose adjustment to overcome the inflammation; however, only a few studies have focused on the effect of these genetic polymorphisms on GC response.

IL-1β: Among the major inflammatory cytokines, IL-1 has a pivotal role. IL-1 is constituted by two distinct polypeptides, IL-1α and IL-1β. IL-1β can promote the production of matrix metalloproteinases and the synthesis of prostaglandins, as well as the production of other inflammatory mediators such as IL-6, IL-8 and TNF-α, thus amplifying the inflammation cascade. IL-1β is therefore a key player in prompting and maintaining inflammation and polymorphisms in the IL-1β gene might be of primary relevance in the modulation of GC response.

A cluster of genes encoding for IL-1α, IL-1β and for the natural receptor antagonist IL-1Ra has been mapped on chromosome 2q13. Polymorphisms in this gene cluster have been associated with a large variety of human diseases and with altered levels of IL-1. So far, the dbSNP build 130 database of NCBI reports 158 submissions for human IL-1, among which, several SNPs are described. Two SNPs in the IL-1β promoter region (C-511T and T-31C) exhibit almost complete linkage disequilibrium. The C-511T SNP results in the loss of AP-2 binding site, while the T-31C (rs1143627) results in the loss of the first T in TATA box. The latter SNP appears to cause a paradoxical increase in IL-1β in the presence of steroids, which could be relevant to the occurrence of GC resistance. Carriers of the haplotype composed of IL-1β -31C allele and -511T allele showed higher plasmatic concentrations of the cytokine, compared to subjects with IL-1β wild-type genotype in Caucasians, but decreased mRNA and protein levels in Japanese subjects. A recent report from this laboratory has investigated the role of the C-511T polymorphism in the response to GCs in 154 young IBD patients, but no relation with GC resistance (14 patients) or dependence (54 patients) was found.

IL-1β is released as an inactive precursor (pro IL-1β, p35) by monocytes and macrophages in response to inflammatory stimuli, and is cleaved to the active mature form (p17) by caspase-1. Caspase-1 is part of a multiprotein cytoplasmic complexes, called NACHT leucine-rich-repeat protein 1 (NALP1) and NALP3 inflammasomes. Jin et al. have recently shown that variants of NALP1 are associated with several autoimmune diseases, and have suggested that mutations in NALP1 gene may result in deregulated secretion of IL-1β. The rs12150220
non-synonymous polymorphism, previously reported to confer susceptibility to autoimmune and autoinflammatory diseases[143] results in the Leu > His amino acidic variation in position 155, between the N-terminal pyrin and NACHT domains of the human NALP1 protein. This region has been highly conserved through primate evolution, which suggests a critical role in protein function[142]. Data from our laboratory have shown that pediatric IBD patients, carriers of the NALP1 homozygote rs12150220 variant genotype, exhibit a higher probability of non-response to GC therapy[23].

TNF-α: TNF-α is a potent pro-inflammatory cytokine released by cells of the immune system upon stimulation, and is almost not detectable in resting conditions[144]. The gene encoding TNF-α is located in the class III region of the major histocompatibility complex within the human leukocyte antigen (HLA) on chromosome 6p21.3[145]. This part of the genome is one of the most polymorphic in humans and contains many genes that encode proteins involved in inflammatory and immune responses[146].

The G-308A (rs1800629) is one of the best documented polymorphisms of the TNF-α gene[147]. This SNP lies in a binding site for the transcription factor AP-1 and the A allele has been shown to have higher transcriptional activity than the G allele, increasing TNF-α production in vitro[148]. The A allele carriers show an enhanced susceptibility to several autoimmune and inflammatory disorders, such as systemic lupus erythematosus, celiac disease[149], Alzheimer disease[150,151], IBD[152], asthma[153] and rheumatoid arthritis[154].

The polymorphism has been correlated also with steroid response in several diseases. In a cohort of 386 pediatric IBD patients, the mutated allele was significantly associated with steroid resistance and requirement for surgery in the subset of 200 CD patients[152]. In addition, previous evidence in a large population of CD patients has shown that this polymorphism is more frequent in steroid-dependent subjects; possibly as a consequence of a more intense TNF-α-driven inflammatory reaction at the mucosal level[155]. The AA genotype has been associated with reduced response to steroids also in other diseases, such as idiopathic nephrotic syndrome[156], rejection episodes in HLA-DR mismatched transplant patients[157] and solid and lymphoid malignancies[158,159].

IL-10: IL-10, known as human cytokine synthesis inhibitory factor, is a cytokine that is produced primarily by monocytes and to a lesser extent by lymphocytes. IL-10 has pleiotropic effects in immunoregulation and inflammation[160-162]. It inhibits the production of inflammatory mediators, and can be considered as a natural immunosuppressant of TNF[163]. GCs upregulate the expression of IL-10[164], and conversely, IL-10 acts synergistically with GCs, improving the ability of DEX to reduce IL-6 secretion in whole-blood cell cultures. In addition, the cytokine increases the concentration of DEX-binding sites in these cells, with no effect on the binding affinity[165]. The human IL-10 gene is located on chromosome 1q31-q32. Previous studies have demonstrated that an A>G polymorphism at nucleotide position -1082 within the IL-10 gene promoter region (rs1800896) influences the cytokine plasma levels, which are significantly higher in patients homozygous for the G allele[166]. No data exist on the role of IL-10 polymorphisms in GC response in IBD, however, the mutated genotype is related to a positive prednisone response in childhood ALL[167]. In accordance, Marino and colleagues[168] have found a better response to GC remission induction therapy in Italian pediatric ALL patients with the mutated allele. In patients affected by rheumatoid arthritis, the high IL-10 producer genotype (-1082GG) is also associated with a favorable outcome, specifically to prednisone therapy[169].

The multidrug resistance gene and other proteins involved in the pharmacokinetics of GCs

Cellular extruding pumps and metabolizing enzymes are the two key pathways involved in the elimination of many drugs. P-gp is one of the major transporters that extrude xenobiotics and drugs out of the cells. The cytochrome P450 (CYP) enzyme superfamily, that catalyzes phase I reactions, is active in the metabolism of many exogenous and endogenous compounds. P-gp and CYP3A are expressed in the intestinal epithelial cells, in lymphocytes and in the liver and have been reported to be involved in the transport and metabolism of GCs.

P-gp: P-gp is a 170-kDa transport membrane glycoprotein, which is responsible for resistance to a number of structurally and functionally unrelated drugs in clinical use. Human P-gp is a phosphorylated and glycosylated protein that consists of 1280 amino acids and two homologous and symmetric sequences, each containing six transmembrane domains and an ATP-binding motif. ATP hydrolysis provides the energy for active drug transport against a steep concentration gradient[165]. The protein plays an important role in the absorption, distribution, and elimination of drugs that are its substrates, among which GCs, and P-gp inhibitors reduce cortisol efflux from human intestinal epithelial cells and T cells[166]. The protein is highly expressed in circulating lymphocytes and epithelial cells from CD and UC patients with GC-resistant IBD[167]. However, a relationship between GC administration and P-gp expression has been described in monocytes from IBD patients[168]. It remains therefore to be clarified if the increased expression of P-gp reflects a primary phenomenon in IBD or a secondary one, influenced by GC therapy.

P-gp is encoded by the ABCB1 (MDR1) gene, located on human chromosome 7q21.12[169], and several studies have demonstrated that genetic polymorphisms in this gene lead to functional alterations and phenotypic variations in P-gp expression[170,171]. The first systematic screening for ABCB1 polymorphisms was performed in 2000[172]; a synonymous SNP in exon 26 (C3435T) was the first variation to be associated with altered protein expression. P-gp expression in the duodenum of individuals with the homozygous variant T allele was decreased when compared with individuals with the C allele (wild-type). Other studies have shown that SNPs at exons 12 (C1236T), 21 (G2677T/A) and 1b (T-129C) may also be associated with altered
transport function or expression\textsuperscript{[172]}\textsuperscript{.} A weak association between GC refractory CD and MDR1 polymorphisms in introns 13 and 16 has been initially described\textsuperscript{[173]}, however, subsequent studies have not confirmed this observation. In 2007, Crucchiara and colleagues studied the C3435T MDR1 polymorphism in 200 pediatric Italian patients with CD and 186 patients with UC treated with GCs, and demonstrated that this SNP was not associated with response to medical therapy\textsuperscript{[174]}. In confirmation of these results, a large study performed on an adult Italian IBD population did not find any association between the polymorphisms of the MDR1 gene (C3435T and G2667T/A) and clinical response to GC therapy\textsuperscript{[175]}. Further studies are therefore needed to clarify if MDR1 polymorphisms play a role in steroid resistance in IBD.

**CYP3A:** CYP3A is the primary CYP subfamily in humans and is responsible for the phase I metabolism of >50\% of drugs currently in use\textsuperscript{[176]}, including GCs\textsuperscript{[177]}. Many drug substrates of CYP3A4 are also substrates of P-gp, and the overlap between CYP3A4 and P-gp is also emphasized by the genomic proximity of these genes (7q22.1 and 7q21.1 for CYP3A4 and MDR1, respectively)\textsuperscript{[178]}. The CYP3A activity of the adult human liver is the sum activity of all subfamily members, including CYP3A5, which is highly polymorphic in Caucasians\textsuperscript{[179]}, and genetic variants may play an important role in inter-individual differences in the pharmacokinetics of drugs metabolized by CYP3A5.

The most frequent and functionally important SNP in the CYP3A45 gene consists of an A6986G transition within intron 3 (CYP3A5*3)\textsuperscript{[180]}, this mutation creates an alternative splice site in the pre-mRNA and results in production of an aberrant mRNA (SV1-mRNA) that contains 131 bp of intron 3 sequence (exon 3B) inserted between exons 3 and 4. The exon 3B insertion turns out in a frameshift and predicted truncation of the translated protein at amino acid 102. Additional intronic or exonic mutations (CYP3A5*5, *6, and *7) may alter splicing and result in premature stop codons or exon deletion\textsuperscript{[181]}.

No report is present in the literature about the role of CYP3A5 polymorphisms in GC resistance in IBD, but plasma prednisolone concentrations, measured by HPLC in 95 renal transplant recipients treated with repeated doses of triple therapy immunosuppression, consisting of prednisolone, tacrolimus and mycophenolate mofetil, have been recently studied\textsuperscript{[182]}. The AUC\textsubscript{0-24} of prednisolone in recipients having both MDR1 3435CC and CYP3A5*3/*3 genotypes tends to be higher than the MDR1 3435TT plus CYP3A5*3/*3 genotype. The CYP3A5*3 polymorphism has also been included in a study to identify pharmacogenomic predictors of outcome in 70 pediatric heart transplant patients followed for at least 1 year post-transplantation, but in this population, no correlation of the CYP3A5*3 SNP with steroid response after transplantation was observed\textsuperscript{[183]}.

**CONCLUSION**

GCs are potent anti-inflammatory drugs and, despite considerable adverse effects, still remain the first-line therapy for inducing a rapid remission in moderate to severe active CD and UC. In addition, high inter-individual variability is observed when these agents are administered to patients with IBD and other chronic inflammatory diseases. A main goal in this area of medicine is therefore to improve the efficacy and safety of these agents and, when possible, to reduce steroid exposure and to employ a non-steroid option. This is particularly important in patients that do not respond, who will suffer considerable steroid-dependent morbidity without any clinical gain.

Pharmacogenomics is a relatively novel branch of investigation and represents an innovative frontier of medicine. Its ambitious goal is to identify genetic determinants that will help physicians in diagnostic decisions, and supply reliable genetic tools to adjust treatment \textit{a priori}. A knowledge of genetic profiles with an impact on drug response would improve cure rates, avoid inadequate regimens or time wasting, and reduce overall health-care costs.

Pharmacogenetic research in IBD has witnessed only modest success; in particular, because treatment response in this disease is influenced by many factors, such as disease duration, behavior and severity, and at present, despite intensive investigation, none of the potential pharmacogenetic markers is strong enough to be used in clinical practice. Reported genetic associations have not yet shown consistent or robust results, and for most of the considered SNPs, results are controversial. More studies with larger sample size and well-characterized patient cohorts, uniformly treated and systematically evaluated, are therefore needed. These studies should support findings with greater statistical confidence that should be hopefully translated into clinical practice in the near future.

**ACKNOWLEDGMENTS**

Raffaella Franca is recipient of a postdoctoral fellowship from IRCCS Burlo Garofolo, Trieste.

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