EXTENDED REPORT

Sulfasalazine sensitises human monocytic/macrophage cells for glucocorticoids by upregulation of glucocorticoid receptor α and glucocorticoid induced apoptosis

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Background: Glucocorticoids (GCs) are commonly used in the treatment of (chronic) inflammatory diseases and cancer, but inherent or acquired resistance to these drugs limits their optimal efficacy. The availability of drugs that could modulate GC resistance is therefore of potential clinical interest.

Objective: To explore the molecular basis of GC sensitisation of GC resistant monocytic/macrophage cells after chronic exposure to sulfasalazine.

Methods: Human monocytic/macrophage THP1 and U937 cells represent a cell line model system characterised by inherent resistance to the GCs dexamethasone and prednisolone. Both cell lines were chronically exposed in vitro to 0.3–0.6 mM sulfasalazine (SSZ) for approximately 3 months, after which they were characterised for GC sensitivity, expression levels of GC receptor and components of the nuclear factor kappa B (NF κ B) signalling pathway, and their ability to undergo GC induced apoptosis.

Results: Chronic exposure to SSZ markedly sensitised both U937 and THP1 cells to dexamethasone (781-fold and 1389-fold, respectively) and prednisolone (562-fold and 1220-fold, respectively). Restoration of GC sensitivity in cells exposed to SSZ was provoked via GC induced apoptosis, coinciding with inhibition of NF κ B activation. Moreover, western blot analysis revealed a markedly increased expression of glucocorticoid receptor α (GR α) in cells exposed to SSZ. Since GR α mRNA levels were only marginally increased, these results suggest that an altered post-transcriptional mechanism was operable which conferred a stable GR α protein on SSZ exposed cells.

Conclusion: These results suggest that chronic targeting of the NF κ B signalling pathway by SSZ may be exploited as a novel strategy to stabilise GR α expression and thereby sensitise primary resistant cells to GCs.

The anti-inflammatory and antiproliferative properties of glucocorticoids (GCs) including prednisolone and dexamethasone have led to their widespread use in the treatment of (chronic) inflammatory diseases such as rheumatoid arthritis (RA) as well as several human cancers (eg, acute lymphoblastic leukaemia).^{1–3} The mechanistic basis for the anti-inflammatory and anticancer effects of GCs involves an interaction with cytosolic glucocorticoid receptor (GR).^{4–5} Upon nuclear translocation, the GC-GR complex can bind to GC responsive elements in the promoter region of several genes which control the expression of both cell death/apoptosis proteins and proinflammatory cytokines such as tumour necrosis factor α (TNF α).^{2–6–7} Moreover, GR can physically interact and antagonise transcription factors, including Activator Protein-1 and nuclear factor kappa B (NF κ B), which facilitate transcription of proinflammatory and antiapoptotic genes.^{4–5–8} At least three isoforms of GR have been reported—GR α , GR β and GR γ ^{9–12}—of which only the α -isoform is capable of high affinity GC binding. The β -isoform lacks the high affinity GC binding capacity and is known as a dominant negative regulator of GR α . The functional and biological significance of GR γ is not yet clear.¹³

The efficacy of GCs can be limited by primary or acquired resistance.^{9–14–19} Several modes of resistance to GC induced apoptosis have been described,^{2–9–17–18–20} including (1) enhanced drug efflux via the multidrug resistance transporter P-glycoprotein, (2) enhanced metabolism by 11 β -hydroxysteroid-dehydrogenase, (3) downregulation of GR expression, (4) an

increased ratio of GR β over GR α expression, (5) post-transcriptional modifications of GR resulting in reduced GC binding affinity, or (6) impaired GC induced apoptosis. Several of these mechanisms have been found responsible for inherent clinical resistance to GCs.^{14–15–21} Elucidation of the molecular basis underlying GC sensitivity and resistance is therefore of key importance in improving the efficacy of GCs for the treatment of both inflammatory and malignant diseases.

In clinical rheumatology the addition of prednisolone to a drug combination of methotrexate (MTX) and sulfasalazine (SSZ), also known as the COBRA combination, appeared to be markedly more effective than SSZ+MTX alone.^{22–24} These observations suggested that SSZ, which inhibits the activation of the transcription factor NF κ B,^{25–27} and MTX are capable of conditioning cells for enhanced prednisolone activity. Recent studies from our laboratory showed that chronic exposure of the human (T lymphocytic) cell line CCRF-CEM to SSZ markedly enhanced its primary sensitivity to dexamethasone (by 10–20-fold).^{28–29} This observation prompted us to investigate whether chronic exposure to SSZ would also provoke restoration of GC sensitivity in myeloid cells with inherent resistance to GCs.

Abbreviations: SSZ, sulfasalazine; GC, glucocorticoid; GR, glucocorticoid receptor; NF κ B, nuclear factor kappa B; IkB, inhibitor kappa B; IKK, inhibitor kappa B kinase; TNF α , tumour necrosis factor α

METHODS

Cell culture

Human THP1 and U937 (monocytic/macrophage) and CCRF-CEM (T lymphocytic) cell lines (ATCC, Manassas, Virginia, USA) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 µg/ml penicillin+streptomycin. Cell cultures were seeded at an initial density of 3×10^5 cells/ml and refreshed biweekly.

Exposure of parental/wild type (WT) U937 and THP1 cells to SSZ was performed essentially as described in detail by De Bruin *et al.*³⁰ Briefly, THP1 and U937 cells were initially incubated with a concentration of SSZ (0.4 mM and 0.3 mM, respectively) that conveyed a 50% growth inhibitory effect. Following 2–3 weeks of adaptation to these SSZ levels, SSZ concentrations were gradually increased to 0.6 mM for both cell lines over a period of another 2.5 months. At this stage, cells had unchanged doubling times and unchanged phenotypic properties compared with parental cells.³⁰ Cells kept at 0.6 mM SSZ (further designated as THP1/SSZ and U937/SSZ) were used for further characterisation of GC sensitivity.

Other procedures

Detailed technical protocols for cell growth inhibition assays, western blot analysis, RT-PCR analysis, assays for apoptosis, NFκB activity assays, chemicals and statistical assays are given in the online supplement available at <http://ard.bmj.com/supplemental>.

RESULTS

Sensitisation of myeloid cells to GCs by chronic exposure to SSZ

Human THP1 and U937 cells are refractory to growth inhibition by the GCs dexamethasone ($IC_{50} > 25 \mu M$) and prednisolone

($IC_{50} > 500 \mu M$) (fig 1). In order to mimic the clinical situation of cellular exposure to dose escalations of SSZ, THP1 and U937 cells were grown in gradually increasing clinically achievable^{31–33} concentrations of SSZ (0.3–0.6 mM) over a period of 3 months.³⁰ The cells were then analysed again for GC sensitivity (fig 1), revealing a three orders of magnitude sensitisation for both THP1/SSZ and U937/SSZ cells for dexamethasone (IC_{50} 0.018 (0.007) µM and 0.032 (0.012) µM, respectively) and prednisolone (IC_{50} 0.41 (0.23) µM and 0.89 (0.07) µM, respectively). For U937/SSZ cells this GC sensitising effect was retained for more than 1 year when cells were grown in the absence of SSZ (data not shown). For THP1/SSZ cells, GC sensitivity was retained for 4 months of cell growth in the absence of SSZ, after which the cells gradually regained their original GC resistance (fig 2A). Interestingly, when these cells were challenged again with SSZ, they rapidly regained their GC sensitive phenotype in <2 weeks (fig 2B).

GC induced apoptosis in cells exposed to SSZ

GCs can exert their antiproliferative effect by inducing apoptosis. Flow cytometric analysis of Annexin-V/7AAD positive cells was performed to determine whether the GC sensitising effect in THP1/SSZ and U937/SSZ cells is the result of induction of apoptosis. Since data were largely similar for THP1 and U937 cells, only those for THP1 cells and its THP1/SSZ subline are shown. Exposure of THP1/WT cells to 10 µM dexamethasone for 24–72 h revealed low numbers (10 (4)%) of apoptotic cells (figs 3A and 4A). This was not due to a lack of apoptotic potential of these cells as treatment of THP1/WT cells for 24 h with 25 µM etoposide (VP-16) resulted in 88 (5)% apoptotic cells (figs 3B and 4A). Dexamethasone induced apoptosis was observed in U937/SSZ and THP1/SSZ cells (44

Figure 1 Reversal of inherent glucocorticoid resistance in THP1 and U937 monocytic/macrophage cells induced by chronic exposure to sulfasalazine (SSZ). Antiproliferative effect after 72 h exposure of (A, B) dexamethasone and (C, D) prednisolone against THP1/WT (open circles), U937/WT cells (open triangles) and their counterparts chronically exposed to SSZ for 3 months (closed symbols). Results of five independent experiments (SD <25%).

Figure 2 Gradual loss of dexamethasone sensitivity in sulfasalazine (SSZ) exposed THP1 cells following withdrawal of SSZ and restoration of dexamethasone sensitivity after repeat challenge with SSZ. (A) Dexamethasone sensitivity after 72 h incubation for SSZ-exposed THP1 cells (solid circles) was assessed after withdrawal of SSZ from the cell cultures for a period of 120 days (solid squares), 220 days (solid triangles) and 270 days (solid diamonds). THP1/WT cells acted as reference (open circles). (B) Dexamethasone sensitivity restored after repeat challenge of SSZ exposed THP1 cells following 270 days of withdrawal of SSZ (fig 2A) (solid diamonds) with 0.6 mM SSZ for a period of 3 days (open squares) and 12 days (open diamonds). Other symbols as in fig 2A. All results shown represent the mean of two experiments performed in duplicate.

(7%) apoptotic cells) after exposure to 1 μM dexamethasone for 72 h, but not as much at earlier time points (figs 3C and 4B). For comparison, GC sensitive human CEM (T lymphocytic) cells showed 22% and 55% apoptotic cells after exposure to 1 μM dexamethasone for 48 h and 72 h, respectively (figs 3D and 4C). In all cases apoptosis induced by VP-16 and dexamethasone was inhibited by the pan-caspase inhibitor ZVAD-fmk. Altogether, these results indicate that chronic exposure to SSZ

induces the ability of initially GC resistant cells to undergo GC induced apoptosis.

Effect of GR expression and changes in the NFκB signalling pathway on GC sensitivity in SSZ exposed cells

To investigate whether the mechanistic basis for the restoration of GC sensitivity in cells exposed to SSZ is associated with

Figure 3 Glucocorticoid (GC) induced apoptosis in THP1 cells exposed to sulfasalazine (SSZ). Effects of dexamethasone (72 h exposure) and VP-16 (24 h exposure) on induction of early apoptosis (Annexin V-FITC staining) and late apoptosis (7-AAD staining) as measured by flow cytometry: (A) THP1/WT cells + dexamethasone; (B) THP1/WT cells + VP-16; (C) THP1/SSZ cells + dexamethasone; (D) CEM cells + dexamethasone. A representative example of three separate experiments is shown.

Figure 4 Time course of glucocorticoid (GC) induced apoptosis in parental and sulfasalazine (SSZ) exposed THP1 cells. Apoptosis induction (% Annexin-V positivity) following 24–72 h exposure to dexamethasone (1–10 μ M) or VP-16 (25 μ M) with or without the apoptosis inhibitor ZVAD-fmk (50 μ M). (A) THP1/WT cells; (B) THP1/SSZ cells; (C) CCRF-CEM cells. Mean (SD) results of three separate experiments.

changes in the expression and/or activity of GR, we first determined whether the GR antagonist RU486 could abrogate this GC sensitisation. RU486 (1 μ M) fully antagonised the growth inhibitory effects of dexamethasone for THP1/SSZ cells (data not shown), supporting a functional role for GR in the observed GC sensitisation effect. Next, mRNA levels of GR α were determined in U937/WT and THP1/WT cells and cells exposed to SSZ. Figure 5A shows that mRNA levels for GR α in U937/SSZ and THP1/SSZ cells were only increased 1.5-fold ($p < 0.01$) and 1.6-fold ($p = 0.02$), respectively, compared with parental cells. GR β mRNA levels in THP1 and U937 cells were at least 200-fold lower than GR α mRNA levels and were unchanged after chronic exposure to SSZ (data not shown). Since NF κ B is a main target of SSZ, we also assessed whether SSZ had a specific effect on the components of this signalling pathway. mRNA levels of NF κ Bp65, the main component of NF κ B, were increased 1.9-fold ($p < 0.001$) and 2.3-fold

($p < 0.001$) in U937/SSZ and THP1/SSZ cells, respectively, compared with parental cells (fig 5B).

Although GR α mRNA levels were only minimally increased, western blot analysis revealed markedly raised levels (5–6-fold) of GR α protein in U937/SSZ and THP1/SSZ cells compared with parental cells in which GR protein was barely detectable (fig 6). Consistent with the GC sensitivity profile and stability of the GC sensitivity phenotype was the finding that increased GR α protein expression was retained in U937/SSZ cells grown without SSZ for 12 months. Moreover, loss of GC sensitivity in THP1/SSZ cells 12 months after SSZ withdrawal (fig 2) correlated with the barely detectable levels of GR α protein in these cells. However, SSZ repeat challenge of these cells upregulated GR α protein expression in parallel with an increase in GC sensitivity. Together these results show that upregulation of GR α protein contributes to the SSZ induced enhancement of GC sensitivity.

Western blot analysis also revealed alterations in the protein expression of NF κ Bp65, its precursor NF κ Bp105 and the NF κ B inhibitory component I κ B α , but not of NF κ Bp50 (fig 6). Increases in NF κ Bp65 and I κ B α protein levels were superimposable with the increases in GR α expression and concomitant GC sensitivity in parental cells, SSZ exposed cells and cells grown in the absence of SSZ.

Finally, to assess whether the imbalance in protein expression of the NF κ B subunits p65 and p50 in cells exposed to SSZ is associated with an inactive state of NF κ B, nuclear extracts of SSZ exposed cells and THP1/WT and U937/WT cells were assayed for NF κ B DNA binding activity. Residual NF κ Bp65 DNA binding activity in THP1/SSZ cells was 15 (8)% compared with THP1/WT cells. Basal levels of NF κ B binding in U937/WT cells were four times lower than in THP1/WT cells and in U937/SSZ cells NF κ B p65 DNA binding was further reduced threefold (not shown).

Collectively, these data indicate that upregulation of the GR α protein together with diminished NF κ B binding activity contribute to the enhanced GC sensitivity seen with chronic exposure to SSZ.

DISCUSSION

This study has shown that chronic exposure to the anti-inflammatory drug SSZ results in restoration of GC sensitivity in two inherently GC resistant monocytic/macrophage cell lines THP1 and U937 by upregulation of GR α levels and consequent GC induced apoptosis.

SSZ is commonly prescribed in the USA and Europe as a disease modifying antirheumatic drug used as second line treatment of patients with RA.^{34–36} It is considered to be a relatively slow acting drug with optimal clinical activity which usually occurs after 4–8 weeks of treatment.^{36–37} The anti-inflammatory properties of SSZ are conveyed via inhibition of the enzyme inhibitor κ B kinase (IKK)- β .^{25–26–33} Inhibition of IKK-mediated phosphorylation of I κ B α , the natural inhibitor of NF κ B, prevents dissociation of the I κ B α /NF κ B complex, thereby abrogating nuclear translocation of NF κ B where it facilitates transcriptional activation of proinflammatory cytokines such as TNF α . Our study indicates that the GC sensitising effects of SSZ in U937 and THP1 cells were not rapidly induced but were only fully apparent after 2–3 months of chronic exposure to SSZ. Interestingly, however, the effects were long lasting in the absence of SSZ and rapidly reinduced when recurrence of GC resistance occurred (fig 2). Thus, direct inhibition of IKK- β by SSZ per se does not seem to be the sole mechanism by which GC sensitivity is provoked and sustained in U937 and THP1 cells.

It is possible that the mechanism of GC resistance in parental U937 and THP1 cells may be related to a low level of GR expression (fig 6) being insufficient to initiate a GC induced

Figure 5 Glucocorticoid receptor (GR) α and nuclear factor kappa B (NF κ B)p65 mRNA levels in parental and sulfasalazine (SSZ) exposed THP1 and U937 cells. mRNA levels of (A) GR α and (B) NF κ Bp65 relative to β_2 -microglobulin (β_2 -M) in parental (WT), THP1/SSZ and U937/SSZ cells determined by real-time PCR. Mean (SD) results of three separate experiments.

apoptotic cascade, in contrast to GC sensitive T lymphocytic CEM cells that express ample levels of GR α .^{18–28} Chronic exposure to SSZ apparently sets conditions under which the GR protein is upregulated or stabilised beyond a threshold level that is sufficient for GC induced apoptosis. Some underlying mechanisms could be instrumental in conferring this SSZ induced GC sensitising effect: (1) TNF α is a known inducer of the dominant negative β -isoform of GR.¹² Consequently, the downregulatory effects on TNF α production induced by SSZ^{30–33} could counteract GR β induction and enhance transcription of GR α . The GR α mRNA levels consistently outweighed those of GR β mRNA in THP1/SSZ and U937/SSZ cells by at least two orders of magnitude. (2) Studies with myelomonocytic cell line models³⁸ and leukaemic blast cells from patients³⁹ indicated that, post-transcriptionally, the stability of GR α , NF κ Bp65 and I κ B α was susceptible to intracellular proteolytic activity of granular serine proteases and the 26S-proteasome/ubiquitination

system.^{38–40–42} Although a direct inhibitory effect of SSZ on serine proteases could not be established,⁴³ our observations that GR α , NF κ Bp65 and I κ B α followed the same pattern of protein up/downregulation after SSZ exposure and withdrawal (fig 6) could be compatible with a common effect on protein stability by reduced 26S-proteasome/ubiquitination activity. (3) Beyond the protein levels of GR α and NF κ Bp65, it is conceivable that transcriptional activity of NF κ B will be impaired in SSZ exposed cells by the direct physical and antagonistic interaction of GR α and NF κ Bp65, as well as by the imbalanced expression of NF κ Bp65 and NF κ Bp50 (fig 6) which requires stoichiometric interactions for optimal transcriptional activity.⁴⁴ The expression of NF κ B controlled survival genes will therefore be impaired, with the result that cells exposed to SSZ will be more prone to GC induced apoptosis (figs 3 and 4).

Although the issue of GC resistance may be less well explored in RA than in haematological cancers, they share common

Figure 6 Expression of glucocorticoid receptor (GR) α , nuclear factor kappa B (NF κ B)p65 and inhibitor kappa B (I κ B) α protein expression in THP1 and U937 cells exposed to sulfasalazine (SSZ). Western blot analysis of GR α , NF κ Bp65, NF κ Bp105, NF κ Bp50 and I κ B α protein expression was evaluated relative to β -actin in parental and SSZ exposed THP1 and U937 cells, SSZ exposed cells (THP1/SSZ) after withdrawal of SSZ and (for THP1 cells) after repeat challenge with SSZ. Lane A: THP1/WT cells; lane B: THP1/SSZ cells; lane C: THP1/SSZ cells (-SSZ 270 days); lane D: THP1/SSZ (-SSZ 270 days + 12 days 0.6 mM SSZ); lane E: THP1/SSZ cells (-SSZ 270 days + 30 days 0.6 mM SSZ); lane F: U937/WT cells; lane G: U937/SSZ cells; lane H: U937/SSZ (-SSZ 270 days). Lanes I and J represent control samples for GR α expression in GC sensitive CEM cells and leukaemic blast cells, respectively. Dexamethasone sensitivity profile (+, sensitive; -, resistant) for each cell line is depicted for comparison. #Protein loading per lane 22.5 μ g; for lanes I and J: 7.5 μ g. A representative picture of two separate experiments is shown.

mechanisms of resistance^{1 2 17 18} and strategies to overcome resistance after disease treatment will be of interest to both diseases. Data from our in vitro studies showed that chronic exposure to SSZ can evoke enhancement of GC sensitivity in primary sensitive cells (T cells)²⁸ and independently in two primary GC resistant myeloid cell lines (this study). These results are in line with empirical clinical observations of enhanced anti-inflammatory efficacy for an SSZ+MTX+prednisolone combination in the treatment of RA.^{22–24 36} and supports further evaluation of SSZ + GC or other NFκB inhibitors + GCs for the treatment of leukaemia. Such combinations would particularly apply to conditions where low levels of GRα expression are considered to be limiting factors in conferring GC sensitivity, but may also be of interest from a GC sparing perspective when targeting primary GC sensitive cells.²⁸ As such, SSZ deserves further exploration in GC based drug combinations in chronic inflammatory diseases such as RA, polymyalgia rheumatica and arthritis temporalis⁴⁵ as well as the leukaemias.³

Further details of the procedures used in this study are given in the online supplement available at <http://ard.bmj.com/supplemental>.

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- Acute myocardial infarction
- MRSA (treatment)
- Bacterial conjunctivitis

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