

Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor

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The glucocorticoid receptor (GR) acts both as a transcription factor itself on genes carrying GR response elements (GREs) and as a modulator of other transcription factors. Using mice with a mutation in the GR, which cannot activate GRE promoters, we examine whether the important anti-inflammatory and immune suppressive functions of glucocorticoids (GCs) can be established in this *in vivo* animal model. We find that most actions are indeed exerted in the absence of the DNA-binding ability of the GR: inhibition of the inflammatory response of locally irritated skin and of the systemic response to lipopolysaccharides. GCs repress the expression and release of numerous cytokines both *in vivo* and in isolated primary macrophages, thymocytes and CD4⁺ splenocytes. A transgenic reporter gene controlled by NF- κ B exclusively is also repressed, suggesting that protein-protein interaction with other transcription factors such as NF- κ B forms the basis of the anti-inflammatory activity of GR. The only defect of immune suppression detected so far concerns the induced apoptosis of thymocytes and T lymphocytes.

Keywords: anti-inflammation/cytokines/innate immune system/lymphocytes/NF- κ B

Introduction

Mammalian organisms have acquired a large repertoire of responses to external attacks (stress responses, immune and inflammatory responses, acute phase response, specialized mobilization of energy stores, repair and wound healing reactions, etc.). Each of these responses needs to be regulated and restricted in magnitude such that homeostasis can be re-established and healthy survival is guaranteed. To give an example, the specific expansion of antigen-triggered lymphocytes cannot be allowed to proceed towards 'leukemic' levels. As an even more drastic example, an uncontrolled response to the invasion of bacteria leads to

septic shock. A particularly important organismic regulatory loop is formed by the release of glucocorticoids (GCs) (Wilckens and De Rijk, 1997). GCs turn off cytokine synthesis and thus protect the organism from undue proliferation and from septic shock. With regard to this function, GCs are in widespread medical use for the treatment of inflammatory disorders such as rheumatoid arthritis, asthma and dermatitis, and autoimmune diseases such as Crohn's disease (reviewed in Karin, 1998).

The effects of GCs are exerted through the glucocorticoid receptor (GR), a ligand-induced transcription factor, which belongs to the nuclear receptor superfamily (Evans, 1988; Beato *et al.*, 1995). GR controls transcription by two major modes of action. One involves binding of GR homodimers to glucocorticoid response elements (GREs) in regulatory sequences of GR target genes. In cell culture experiments, a second mode of action has been identified: GR modulates the activity of other transcription factors such as AP-1, NF- κ B and Stat5, independently of direct DNA contact, a process designated as cross-talk (Jonat *et al.*, 1990; Stöcklin *et al.*, 1996; reviewed in Beato *et al.*, 1995; Herrlich, 2001). The interference with the activities of these other transcription factors appears to occur at a late stage of transcriptional initiation, after formation of the pre-initiation complex (Nissen and Yamamoto, 2000; Herrlich, 2001). The GR itself does not need to bind to DNA for this second mode of action. In fact, GR mutants in the transactivation domain or in the dimerization domain (D-loop), which cannot activate GRE promoters, are perfectly proficient in cross-talk (Caldenhoven *et al.*, 1995; Heck *et al.*, 1994, 1997).

We have recently established a mouse model which permits exclusive study of the cross-talk function in the absence of GR-dependent gene transcription (Reichardt *et al.*, 1998). By gene targeting, the point mutation A458T was introduced into the GR D-loop (homozygotic GR^{dim} mice). In these mice, AP-1-mediated gene expression is repressed efficiently by GCs in the absence of transcriptional activation of classical GRE-regulated genes (Reichardt *et al.*, 1998; Tuckermann *et al.*, 1999). Here, we have exploited GR^{dim} mice to supply the first *in vivo* proof that local and systemic inflammatory responses are repressed potently by GR in the absence of DNA binding. The basis for the anti-inflammatory activity of the DNA binding-defective GR appears to be its normal ability to repress inflammation-relevant genes in various cell types, predominantly by negative interference with NF- κ B activity.

Results and discussion

Inflammatory responses are repressed efficiently by glucocorticoids in mice carrying a DNA binding-defective GR

A frequently used model of acute inflammation is phorbol ester (phorbol 12-myristate 13-acetate; PMA)-induced

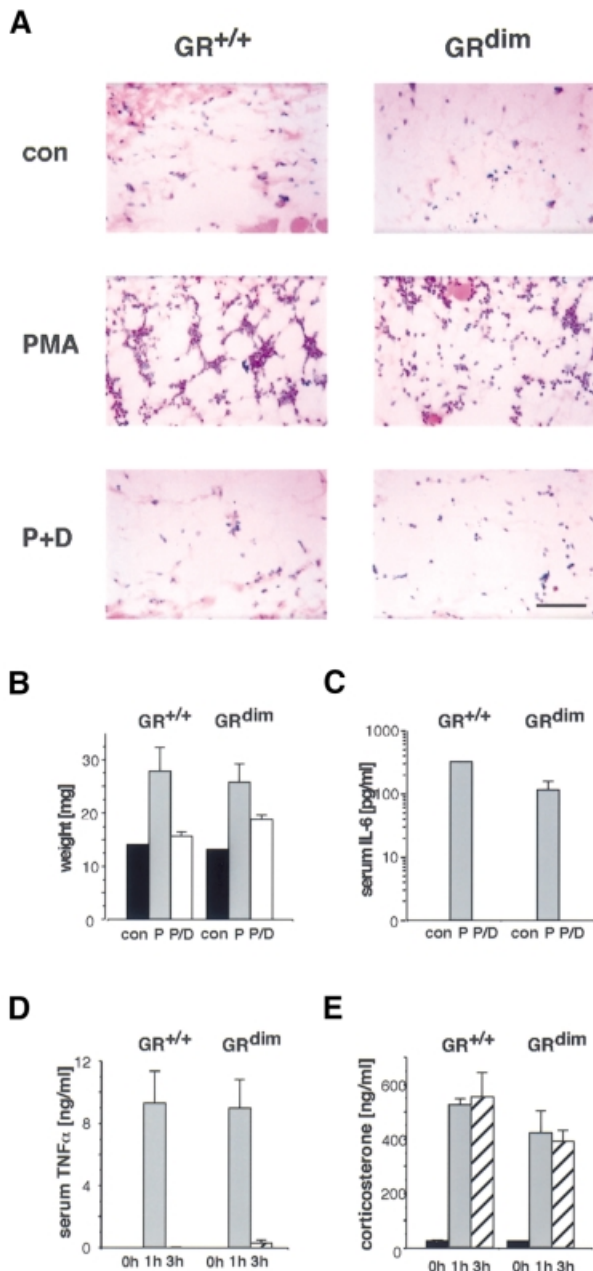


Fig. 1. GCs potently suppress local and systemic inflammatory responses in GR^{dim} mice. (A) Hematoxylin–eosin-stained sections of subdermal fat tissue derived from the back skin of wild-type (GR^{+/+}) and GR^{dim} mice treated with either vehicle acetone (con), 10 nmol PMA (P) or PMA plus 50 µg dexamethasone (P+D) for 6 h. Scale bar: 100 µm. (B) Vehicle acetone (con), 1 nmol PMA (P) or PMA plus 5 µg dexamethasone (P/D) were applied ectopically to the ears of the mice and the swelling measured after 6 h. (C) IL-6 levels in serum of mice treated as described in (A) were determined by ELISA. (D and E) Wild-type (GR^{+/+}) or GR^{dim} mice were injected with 100 µg of LPS per mouse and killed at the time points indicated. TNF-α serum levels were measured by ELISA (D) and corticosterone serum levels by RIA (E).

oedema formation (Gschwendt *et al.*, 1984; Lloret and Moreno, 1995). Application of PMA to the skin causes swelling associated with increased vascular permeability and rapid influx into the skin of neutrophilic granulocytes and mononuclear cells. This inflammatory response can be inhibited efficiently by topical application of GCs. To

address the question of whether this repressive effect is retained in mice with a DNA binding-defective GR, we analysed the response of PMA-irritated skin to GC treatment. Topical application of PMA to the dorsal skin of either wild-type or GR^{dim} mice induced rapid infiltration of inflammatory cells into the fat tissue underlying the dermis, exhibiting the features of panniculitis (Figure 1A). Simultaneous treatment with the synthetic GC dexamethasone almost completely inhibited the panniculitic phenotype in mice of both genotypes.

To quantitate the inhibition of swelling, PMA was also applied to the ears. This topical treatment led to a substantial increase in weight due to massive oedema formation, in both wild-type and GR^{dim} mice (Figure 1B). In the presence of GC, this increase was strongly reduced, although slightly less efficiently in GR^{dim} mice than in wild-type mice.

PMA-induced local inflammation of the skin also led to a marked systemic response, as demonstrated by an elevation of the interleukin-6 (IL-6) serum levels (Figure 1C). The secretion of IL-6 into the blood was completely prevented by GC. In accordance with these data, the synthesis of IL-6 mRNA and the expression of the NF-κB-dependent E-selectin gene, which facilitates rolling of immune cells (Brostjan *et al.*, 1997), was inhibited efficiently in the skin of wild-type and GR^{dim} mice (data not shown). Taken together, these findings demonstrate that the topical anti-inflammatory activity of GCs as employed in the treatment of numerous skin disorders, as well as inhibition of the release of acute phase mediators, are established predominantly by the DNA binding-independent function of GR.

To mimic a bacterial infection with subsequent systemic inflammatory response, we injected lipopolysaccharides (LPS) into wild-type and GR^{dim} mice. The response elicited, typical for endotoxic shock, can be monitored by the release of cytokines into the blood. Physiologically the response is then terminated by the action of endogenous GCs, which are released after activation of the hypothalamus–pituitary–adrenal (HPA) axis, and therefore abolished in adrenalectomized or hypophysectomized animals due to their inability to execute a corticosterone response (Zuckerman *et al.*, 1989). To study the response, we followed secretion of the cytokine tumour necrosis factor-α (TNF-α) over time and found that TNF-α was massively induced at 1 h after LPS injection in both wild-type and mutant mice. The levels returned to basal values after 3 h (Figure 1D). In parallel, the serum concentrations of corticosterone were up-regulated (Figure 1E). The magnitude and kinetics of both TNF-α and corticosterone serum levels were similar in mice of both genotypes. This clearly indicates that DNA binding by GR is not required for terminating a systemic inflammatory response as induced by LPS injection.

The GR^{dim} receptor represses cytokine gene expression in primary immune cells

Complex inflammatory responses such as the phorbol ester-induced oedema of the skin or endotoxic shock after LPS injection involve multiple cell types and the production of a variety of inflammatory mediators (Barnes, 1998). For instance, macrophages and T lymphocytes release cytokines. The sum of these reactions forms the

pre-conditions for the development of innate and adaptive immunity. Inhibition by GCs occurs on several levels: removal of cells by apoptosis which could result from the reduction of intracellular components required for survival or from direct induction of a proapoptotic gene product; and inhibition of effector functions, e.g. the synthesis of cell surface proteins, of cytokines or proteins involved in invasion of immune cells into tissues (Barnes, 1998). We tested several of these parameters in our mutant mice.

The potency of the DNA binding-defective GR to repress cytokine production in the living animal was explored by measuring the expression of cytokines in primary macrophages and lymphocytes. Peritoneal macrophages were isolated after thioglycolate treatment and cultured for 24 h prior to stimulation by LPS. mRNAs transcribed from the cytokine genes TNF- α , IL-6 and IL-1 β were found to be up-regulated after 2 h of LPS treatment (Figure 2A–D). Regardless of the genotype, this induction was strongly inhibited when cells had been pre-treated with dexamethasone. In addition to cytokines, the release of prostaglandins is indicative of an inflammatory process. mRNA induction of the rate-limiting cyclooxygenase-2 gene (*Cox-2*) by LPS could be blocked efficiently by dexamethasone in macrophages of GR^{dim} and wild-type mice (Figure 2E). Similarly, effective repression was observed for PMA-induced transcription in mouse embryonic fibroblasts (MEFs; data not shown).

GCs inhibited the expression of T-cell cytokines. To this end, primary thymocytes from wild-type and GR^{dim} mice were treated with PMA/ionomycin (P/I) as a model for antigen-induced activation of T cells. P/I application led to strong induction of IL-2 and interferon- γ (IFN- γ) mRNA expression, whereas simultaneous treatment with dexamethasone suppressed the P/I-induced levels in both types of mice to a similar extent (Figure 3A–D). Finally, CD4⁺ splenocytes were analysed as an example of mature T lymphocytes. T-cell receptor ligation by α CD3 caused induction of IL-2 mRNA expression, which was completely prevented by dexamethasone treatment (Figure 3E). No difference in the ability to suppress T-cell activity was found between wild-type and GR^{dim} splenocytes.

Cycloheximide experiments indicated that the effects of GCs were direct (not shown), which is compatible with the properties of the mutant GR. DNA binding by GR is obviously dispensable for the repression of *Cox-2* transcription and that of various cytokine genes in all primary cells tested.

Glucocorticoids repress pro-inflammatory NF- κ B activity in the absence of GR DNA binding

The expression of most pro-inflammatory genes, including those investigated here, depends on the activation of NF- κ B (Baeuerle and Baltimore, 1996). NF- κ B most probably represents the major target of GR in the inhibition of an inflammatory response. Two modes of repression of NF- κ B activity have been described: (i) inhibition occurs by protein–protein interaction between promoter-bound NF- κ B and GR (Caldenhoven *et al.*, 1995; Heck *et al.*, 1997; Nissen and Yamamoto, 2000); and (ii) GCs induce the transcription of I κ B α resulting in sequestering nuclear NF- κ B into the cytoplasm, thereby abolishing transcription (Auphan *et al.*, 1995; Scheinman *et al.*, 1995). In the mutant mice experiments described

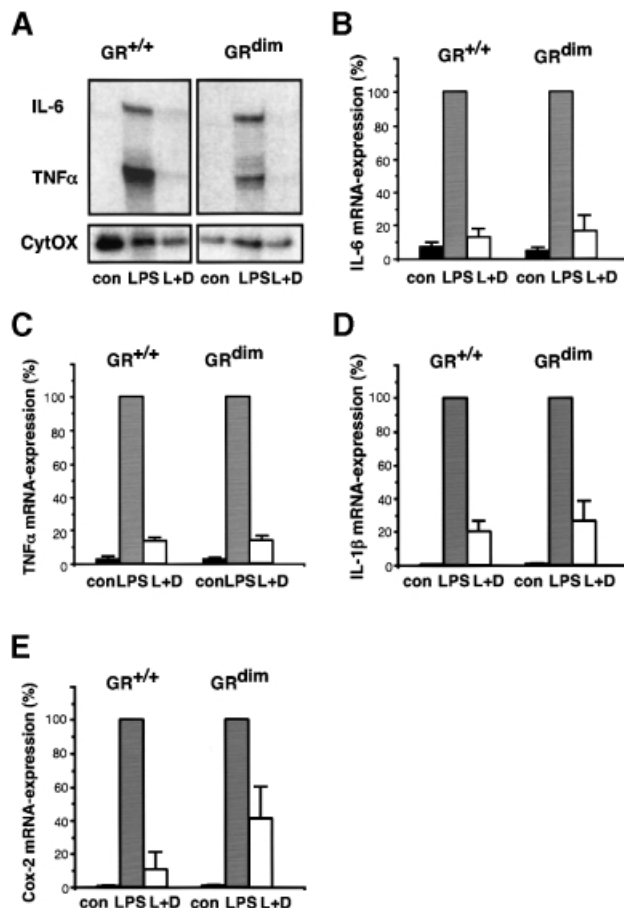
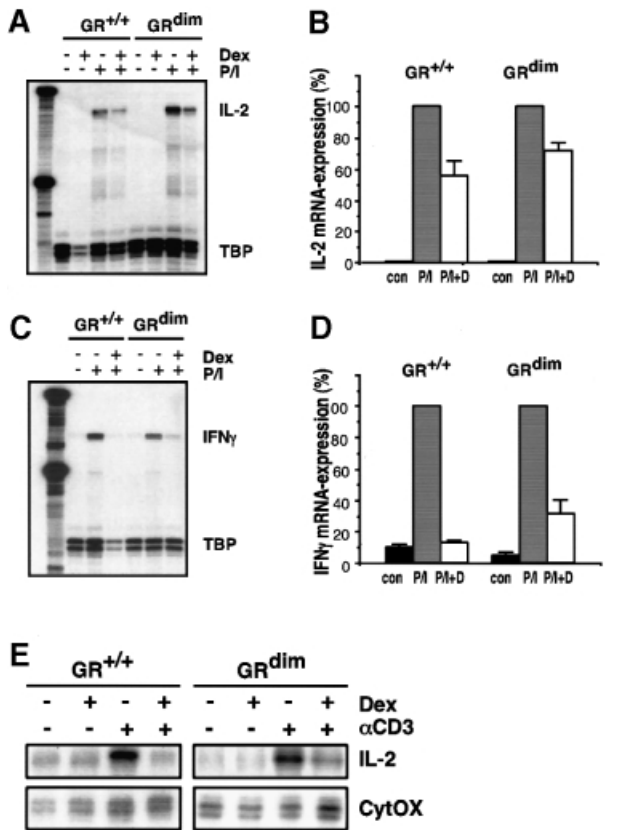


Fig. 2. Repression of cytokine genes by GC in peritoneal macrophages does not require the DNA-binding function of GR. (A) TNF- α and IL-6 mRNA levels were determined by RNase protection assay in peritoneal macrophages of either wild-type (GR^{+/+}) or GR^{dim} mice cultured under the following conditions: mock-treated (con), treated with 100 ng/ml LPS for 2 h (LPS) and LPS treatment in the presence of 10⁻⁶ M dexamethasone (L+D). Dexamethasone was added 1 h prior to LPS. mRNA levels for CytOx (cytochrome oxidase) were used for normalization. (B and C) Quantitative evaluation of the data in (A). The levels after induction by LPS were taken as 100%. mRNA levels for IL-1 β (D) and cyclooxygenase-2 (*Cox-2*) (E) were determined by quantitative PCR using HPRT for normalization.

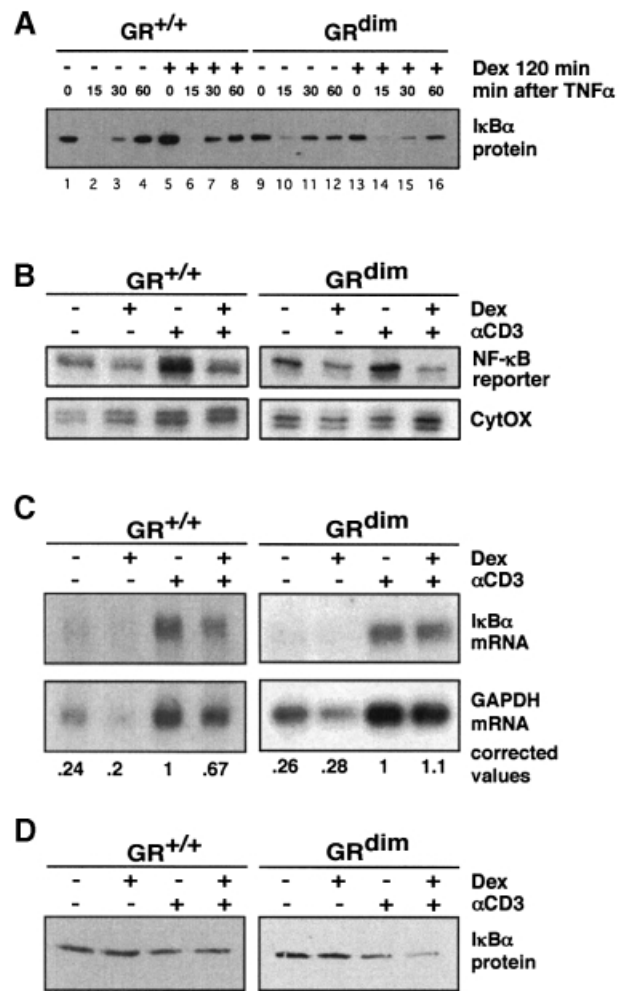
here, a direct GRE-dependent transcription of I κ B α does not seem to play a major role as the mutant GR cannot activate genes and yet the inflammatory response is inhibited. One could, however, imagine the existence of a mode of I κ B α induction not requiring direct GR–DNA binding. Such GR action has been found for the synergy with Jun homodimers (Diamond *et al.*, 1990; Teurich and Angel, 1995). We therefore investigated the fate of I κ B α and NF- κ B activity in GR^{dim} mice.

The expression of I κ B α was measured in MEFs and splenic CD4⁺ cells. Primary MEFs isolated from both wild-type and GR^{dim} mice responded to treatment with TNF- α with disappearance and then delayed resynthesis of I κ B α protein. In wild-type cells, but not cells from GR^{dim} mice, GC treatment caused >2-fold increases of I κ B α mRNA (data not shown) and protein beyond basal levels (Figure 4A, compare lanes 1 and 5 for wild-type and lanes 9 and 13 for GR^{dim}), also reflected in earlier resynthesis (Figure 4A, compare lanes 3 and 7). I κ B α transcription thus appears to depend on proper GR–GRE



binding or on an unknown reaction requiring GR dimer formation. In electrophoretic mobility shift assay (EMSA) experiments, the same cells of both genotypes showed identical kinetics for the formation and stability of NF- κ B–DNA complexes after stimulation with TNF- α , both with and without treatment with GCs (not shown), compatible with the idea that inhibition occurs after binding of NF- κ B to DNA.

Splenic $CD4^+$ cells were isolated from both wild-type and GR^{dim} mice into both of which we had crossed a transgene reporter carrying three NF- κ B-binding sites as the only promoter elements in front of the β -globin TATA box (Lernbecher *et al.*, 1993). This setting permits analysis of IL-2 transcription, I κ B α and exclusive NF- κ B activity in the same primary cells. Stimulation of the cells with α CD3 led to a marked increase of mRNA expression of the reporter (Figure 4B) and of IL-2 (not shown), indicating induction of NF- κ B activity. Concomitant addition of GC repressed reporter expression (as well as IL-2) equally well in both genotypes (Figure 4B), demonstrating that NF- κ B activity is fully inhibited by GR in the absence of its DNA-binding function. Interestingly, in $CD4^+$ cells



$GR^{+/+}$ and GR^{dim} MEFs were treated with 10^{-7} M dexamethasone for 2 h followed by treatment with 50 ng/ml TNF- α . I κ B α protein content was measured by immunoblotting. (B) $CD4^+$ splenocytes from NF- κ B transgenic reporter mice expressing either wild-type ($GR^{+/+}$) or DNA binding-defective GR (GR^{dim}) were cultured on α CD3-coated culture dishes in the absence or presence of 10^{-6} M dexamethasone for 4 h. Expression of the reporter gene (B) was determined by RNase protection analysis and normalized to CytOX. Relative I κ B α mRNA levels (C) were determined by northern blot analysis and I κ B α protein (D) was detected by western blot analysis.

I κ B α mRNA and protein were not induced by GC alone (Figure 4C and D), perhaps suggesting a cell type-specific contribution to its regulation. The levels achieved upon α CD3 treatment were somewhat inhibited by the presence of dexamethasone. These data further confirm that the inhibition of NF- κ B does not depend on elevated I κ B α .

Taken together, the results obtained in MEFs and $CD4^+$ splenocytes demonstrate that efficient repression of NF- κ B activity occurs in the absence of DNA binding by GR and that induction of I κ B α by GC does not play a significant role in conferring GC-dependent repression of NF- κ B activity under the conditions tested here. These results are in agreement with the finding that GCs do not interfere with the formation of a pre-initiation complex at an NF- κ B-dependent promoter but rather trigger a subsequent

step of transcriptional initiation (Nissen and Yamamoto, 2000).

The reduction of cytokine release upon GC treatment may well suffice to bring dependent cells into apoptosis. The ability of GCs to induce apoptosis is indeed used for the treatment of leukemia. In cell culture, a transactivation-defective GR with non-disturbed inhibitory activity on AP-1 could indeed block the proliferation of appropriately designed Jurkat cells (Helmberg *et al.*, 1995). However, peripheral T lymphocytes, thymocytes (Reichardt *et al.*, 1998) and thymocytes in fetal thymus organ culture (not shown) were resistant, indicating that a GR dimer-specific gene programme was needed to bring these cells into apoptosis. The difference from leukemic cells may be that the survival of these primary cells has not yet become cytokine dependent. For the anti-inflammatory action of GCs *in vivo*, induced apoptosis thus does not seem necessary or limiting.

Conclusions

GCs are among the most widely employed anti-inflammatory drugs, although long-term application is accompanied by massive side effects. Thus, it is conceivable that the search for improved drugs is a major challenge for pharmacological research. The finding that GR mediates the effects of GCs by two different modes of action has suggested that interfering with one of these may provide a tool to develop new anti-inflammatory compounds. Since DNA binding is abrogated in GR^{dim} mice, these animals are the ideal model to determine the contribution of protein-protein interaction of GR to the anti-inflammatory action of GCs *in vivo*. In this study, we have shown that inflammatory responses in the living animal are repressed efficiently in the absence of DNA binding. These results strongly suggest that this repression is due, at least in part, to the unimpaired ability of the GR^{dim} receptor to inhibit pro-inflammatory protein production by macrophages and T lymphocytes, through negative interference with NF- κ B and possibly other pro-inflammatory transcription factors. The reduction of cytokines may also explain why B cells of GR^{dim} mice enter apoptosis (our unpublished data). A separate pro-apoptotic programme requiring GR DNA binding exists in thymocytes and mature T cells. Our findings suggest that ligands selectively acting via the DNA binding-independent function of GR should suffice for the treatment of inflammatory diseases. Such ligands would possibly help to avoid the adverse side effects of long-term treatment, such as GC-induced osteoporosis, growth retardation, redistribution of fat, muscle degeneration and, when topically applied, atrophy of the skin.

Materials and methods

RNA analysis

Total RNA was isolated after guanidinium isothiocyanate extraction according to standard procedures. RNase protection assays and cDNA synthesis were done as described previously (Reichardt *et al.*, 1998). RNase protection assays were quantified using a phosphoimager. Quantitative PCR was performed using a Lightcycler System according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) using the following primers: 5'-CATTATGCCGAGGATTTG and 5'-TGGGGCTGTACTGCTTA for the amplification of a 389 bp fragment of hprt, 5'-GCAAACGCTTCTCCCTGAAG and 5'-CGCTTG-CATTGATGGTGGCTG for the amplification of a 389 bp fragment of

cox-2, and 5'-TCCTGAACTCAACTGTGA and 5'-CCAGCAGGTTAT-CATCAT for amplification of a 469 bp fragment of IL-1 β .

Protein analysis

For western blotting analysis, proteins were extracted from cells in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.5 mM sodium vanadate, leupeptin (10 μ g/ml), 1% (v/v) NP-40 and 10% (v/v) glycerol, and separated on a 12% SDS-polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane, hybridized with a rabbit I κ B α antiserum (kindly provided by Dr L.Schmitz, Heidelberg, Germany) and immunoreactive bands visualized by enhanced chemiluminescence.

Isolation and cultivation of primary cells

MEFs were isolated from day 14.5 embryos as described previously (Reichardt *et al.*, 1998). The CD4⁺ subpopulation of splenic lymphocytes was isolated by magnetic bead selection and cultured on anti-CD3-coated culture dishes (mAb145-2C11, 1 μ g/cm²). Primary thymocytes were isolated as described previously (Reichardt *et al.*, 1998) and cultured at a concentration of 10⁶ cells/ml. To isolate macrophages, mice were injected intraperitoneally with 1.5 ml of sterile thioglycollate medium 4 days prior to the experiment. Macrophages were harvested by peritoneal lavage with 2 ml of sterile phosphate-buffered saline, centrifuged and resuspended in RPMI medium supplemented with 10% fetal calf serum (FCS). The cells were seeded at a concentration of 10⁶ cells/ml in 24-well plates, incubated for 24 h at 37°C and washed to remove non-adherent cells. During the experiment, FCS was omitted.

Hormone determination

Serum was obtained by centrifugation of freshly isolated EDTA-blood at 5000 r.p.m. for 10 min. The concentration of TNF- α and IL-6 was determined by enzyme-linked immunosorbent assay (ELISA) and the level of corticosterone by radioimmunoassay (RIA) using commercially available kits (Endogen, Woburn, USA and ICN Biomedicals, Meckenheim, Germany).

Animal experimentation

To study systemic inflammation, mice were injected intraperitoneally with 100 μ g of LPS, killed by CO₂ and the blood collected for cytokine and hormone determination.

To induce local inflammation in the dorsal skin, mice were shaved 4 days before experimentation and treated topically with 10 nmol PMA or in combination with 50 μ g of dexamethasone dissolved in 200 μ l of acetone as described (Tuckermann *et al.*, 1999). The animals were killed 6 h after application and both skin biopsies and the blood were collected for histological analysis and cytokine measurement. The ear oedema formation assay (Gschwendt *et al.*, 1984) was performed by ectopic application of the vehicle acetone, 1 nmol PMA or PMA plus 5 μ g of dexamethasone to the ear of the mice. After 6 h, a defined area of the ear was excised and the weight determined.

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