Down-regulation of Human Granzyme B Expression by Glucocorticoids

Dexamethasone inhibits binding to the Ikaros and AP-1 regulatory elements of the granzyme B promoter*

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The serine protease granzyme B is an essential component of the granule exocytosis pathway, a major apoptotic mechanism used by cytotoxic T lymphocytes and natural killer cells to induce target cell apoptosis. Granzyme B gene transcription is induced in activated lymphocytes upon antigenic stimulation, and several regulatory regions including CBF, AP-1, and Ikaros binding sites have been shown to be essential in the control of granzyme B promoter activation. Dexamethasone, a glucocorticoid that is widely used as an immunomodulatory and anti-inflammatory agent, inhibits granzyme B mRNA transcript in phytohemagglutinin-activated peripheral blood mononuclear cells. Transfection of a reporter construct containing the −148 to +60 region of the human granzyme B promoter demonstrated that this region was the target for dexamethasone repression. Mutation of Ikaros or AP-1 binding sites in the context of the granzyme B promoter demonstrated that both sites participate in dexamethasone-mediated inhibition of the granzyme B promoter activity. Electromobility shift assay revealed that dexamethasone abolished the binding of nuclear transcription factors to the Ikaros binding site and reduced AP-1 binding activity. These results indicate that dexamethasone is able to abrogate the transcriptional activity of the human granzyme B gene promoter by inhibiting the binding of nuclear factors at the AP-1 and Ikaros sites.

Graft rejection remains a significant obstacle to successful allogeneic transplantation. Understanding the molecular mechanisms responsible for allograft rejection is essential in developing new therapeutic approaches that will lead to successful allogeneic transplantation. The immune effector cells responsible for this clinical syndrome are cytotoxic T lymphocytes and natural killer cells. Antigen-activated T-cell activation involves a variety of molecular transduction signals that lead to the acquisition of a killing potential by these cells. Two major pathways lead to the cell death of target cells by cytotoxic T lymphocytes: the Fas/FasL interaction system, which delivers a complement-like death signal, and the granule exocytosis pathway, a major apoptotic mechanism used by cytotoxic T lymphocytes and natural killer cells. Upon specific recognition and T-cell activation by foreign antigens present on target cells, cytotoxic T lymphocytes and natural killer cells accumulate granules in their cytoplasm. These granules contain the pore-forming protein perforin, which undergoes calcium-dependent polymerization on the target cell membrane, thereby forming a complement-like lesion (1) that facilitates delivery of the granule constituents, including granzymes, to the target cell cytoplasm. Delivery of granule component proteins is required for the induction of apoptosis (2–4). Serine protease granzyme B is an essential mediator of early DNA fragmentation within the target cell in this pathway (5) and elicits apoptosis in target cell by initiating caspases activation (6–8).

Targeted deletion of granzyme B and perforin genes in mice also demonstrated their role in graft rejection and graft versus host disease (9–11). In humans, the presence of activated T-cells expressing granzyme B and perforin has been observed in acute heart (12), lung (13), and kidney allograft rejections (14, 15).

Disease states in which T-cell and cytokine-mediated tissue damage predominate, including allograft rejection, autoimmune disease, and many cases of chronic inflammation and delayed hypersensitivity, are effectively treated with glucocorticoids. Glucocorticoids have immunosuppressive and anti-inflammatory properties and affect the growth, differentiation, and function of monocytes and lymphocytes. The molecular basis of the immunosuppressive effects of glucocorticoids is not clearly understood.

Glucocorticoids modulate the production of various cytokines, including IL-116, IL-217–20, IL-421, IL-522, IL-823, 24, interferon γ25, and tumor necrosis factor α25, and can exert their effect by interfering with the action of transcription factors. Glucocorticoids have been shown to repress AP-1-mediated transcriptional activation (26) and to impair NF-κB activation (27–30). NFAT, the nuclear factor of activated T-cells, has been described as a possible target for dexamethasone in thymocyte apoptosis (31).

In a previous study, we observed that granzyme B expression could be down-regulated at the protein level in graft-infiltrating lymphocytes in heart-transplanted patients with severe and mild rejection under corticoid treatment (32). This effect, which could be reversible, coincided with graft stabilization and preceded the complete disappearance of cellular infiltration (32). Similarly, in kidney transplantation, it has been reported that effective antirejection therapy resulted in rapid

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1 The abbreviations used are: IL, interleukin; PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cell; Dex, dexamethasone; NF, nuclear factor; CAT, chloramphenicol acetyltransferase.
Down-regulation of Granzyme B Transcription by Dexamethasone

In this study, we have examined the molecular basis of the previously mentioned down-regulation of lytic protein expression by glucocorticoids. In both humans and mice, the granzyme B gene is not expressed in resting T-cells but is induced at the transcriptional level 24 to 72 h after T-cell activation (33, 34). The DNA regulatory element that controls the level of granzyme B gene transcription in activated T-cells resides between nucleotides −148 and +60 (relative to the transcriptional start point +1) of the human granzyme B gene promoter (35). This proximal promoter region contains Ikaros and AP-1/CBF binding sites, previously shown to be essential in human and mouse granzyme B promoter activation for T-cell stimulation (36–38).

In this report, we examine the effects of the glucocorticoid hormone dexamethasone on the transcriptional activity and DNA binding pattern of the human granzyme B gene promoter in PHA-activated peripheral blood mononuclear cells (PBMCs). We present evidence that dexamethasone significantly decreases the activation of granzyme B transcription in these PHA-activated cells. The −148 to +60 region of the granzyme B promoter is identified as a target for this suppression. Electrophoretic mobility shift assays revealed that dexamethasone abolished the binding of nuclear transcription factors to the Ikaros and AP-1 sites of the promoter, whereas CBF binding remained unaffected. We show here for the first time that dexamethasone has an inhibitory effect on the binding of nuclear factors to the Ikaros and AP-1 regulatory elements of the human granzyme B promoter in PHA-activated PBMCs.

EXPERIMENTAL PROCEDURES

Cell Culture—A buffy coat of human PBMCs was purified by Ficoll-Hypaque density centrifugation. PBMCs were cultured in complete RPMI 1640 (Life Technologies, Inc.) supplemented with 10% inactivated human serum and activated with PHA at a final concentration of 15 μg/ml, 2 h before the addition of dexamethasone at various concentrations (1 μM, 100 nM, and 10 nM).

RNA Isolation and Northern Blot Hybridization—Total cellular RNA was extracted from peripheral blood mononuclear cells in guanidinium isothiocyanate according to the method of Chomczynski and Sacchi (39). After electrophoresis of 10 μg of total RNA from each sample on a 1% agarose formaldehyde gel, transfer to a Hybond N nitrocellulose membrane was carried out (Amersham Pharmacia Biotech). A granzyme B 32P-labeled RNA probe was prepared using the SP6 RNA polymerase as described previously (40). Hybridization was carried out with this probe and RNA probe was prepared using the SP6 RNA polymerase as described above. Three or four independent experiments were performed in duplicate on PBMCs obtained from four healthy blood donors. After 48 h of culture, 5 × 10^6 PHA-activated cells were transfected by electroporation using 4 μg of plasmid. Ten-μl aliquots were measured for 10 s following the injection of luciferase reagent as recommended by the manufacturer (luciferase assay, Promega) using a luminometer (Berthold Systems, Inc.). Three independent experiments were performed in duplicate on PBMCs obtained from three healthy blood donors.

Western Blotting Analysis—Twenty μg of nuclear extracts were prepared as described previously (36), and whole extracts were prepared by the Sambrook method (44) from PHA-activated PBMCs after 48 h of culture either without or with various concentrations of dexamethasone (1 μM, 100 nM) and loaded onto 10% SDS-polyacrylamide gels. Proteins were blotted onto a nitrocellulose membrane (BAS53, Sera Labo) and blocked overnight with 5% nonfat milk/phosphate-buffered saline, 3% Tween 20. Membranes were incubated for 1 h with a 1/1000 dilution of a purified anti-Ikaros serum (45), then washed and revealed using goat anti-rabbit IgG peroxidase conjugate (1/5000, 1 h). Peroxidase was revealed with an Amersham Pharmacia Biotech ECL kit. Proteins were quantified before being loaded onto the gel, and equal loading of extracts with and without dexamethasone was verified by Ponceau staining.

RESULTS

Dexamethasone Partially Abrogated the Stimulatory Effect of PHA on Human Granzyme B mRNA Expression—Granzyme B mRNAs expression was studied by Northern blot analysis of total RNA samples extracted from unstimulated and PHA-stimulated PBMCs. As expected (34), no expression was found in resting cells, whereas granzyme B mRNA was present with a mobility of 1.5 kb in PHA-stimulated cells (Fig. 1). The addition of dexamethasone abrogated the induction of human granzyme B messenger RNA expression, which was observed after PHA stimulation of PBMCs (Fig. 1). CD8+ T lymphocytes, which expressed a higher granzyme B level than CD4+ T lymphocytes, are highly sensitive to dexamethasone-induced apoptosis (46). To elimi-
Fig. 1. Inhibition of granzyme B RNA expression by Dex. Total RNA from resting PBMCs, PHA-stimulated PBMCs, and PHA-stimulated PBMCs treated with 1 μM of Dex were successively hybridized with a granzyme B riboprobe (see “Experimental Procedures”) and β-actin cDNA.

Fig. 2. Dexamethasone inhibits the PHA-mediated transactivation of the human granzyme B promoter. PBMCs were activated with PHA at a final concentration of 15 μg/ml with or without Dex at different concentrations (1 μM, 100 nM, and 10 nM). After 48 h of culture, 10^6 cells were transfected with 10 μg of the plasmid CAT3A or PBLCAT3. CAT activity was assayed 24 h later. The percentage of activation relative to the CAT3A activity in the absence of dexamethasone (taken as 100%) represents the mean values ± S.E. of at least four individual experiments in duplicate. The CAT3 is vector without promoter.

Dexamethasone-mediated inhibition of granzyme B promoter activation. The Dex treatment and the cotransfection experiment with c-Jun cannot reverse glucocorticoid-mediated repression of CAT2AM2 (77), corresponding to mutation on the AP-1/CBF binding site with an intact AP-1/CBF binding site upstream of the SV40 promoter in the pGL3 vector (data not shown).

data suggest that dexamethasone-induced down-regulation of granzyme B transcription because of apoptosis of CD8+ cells, the CD4+/CD8+ ratio before and after dexamethasone treatment was analyzed by cytofluorometry. No difference in this ratio could be demonstrated (data not shown).

PHA Activation of the Granzyme B Promoter Was Down-regulated by Dexamethasone in Primary Human PBMCs—To determine whether one of the possible mechanisms of dexamethasone inhibition of granzyme B mRNA induction involved a change in the transcriptional activity of the human granzyme B promoter, we transiently transfected PHA-activated PBMCs with a vector in which the CAT reporter gene transcription was under the control of the human granzyme B gene proximal promoter (−148 to +60) (35, 36). The CAT activity driven by the reporter fragment was significantly inhibited by treatment with three different concentrations of dexamethasone (1 μM, 100 nM, and 10 nM) (Fig. 2). Dexamethasone was thus found to inhibit granzyme B promoter activation in a dose-dependent manner.

Binding of Nuclear Proteins from Stimulated Primary Human PBMCs to the Ikaros GB1 and AP-1 GB2 Sites of the Granzyme B Promoter Was Inhibited by Dexamethasone—We investigated whether dexamethasone inhibited granzyme B gene transcription by interfering with nuclear proteins that bind to the human granzyme B promoter. We have previously shown that the Ikaros binding site GB1 (−143 to −114) and the AP-1/CBF binding site GB2 (−103 to −77) were essential for the activation of transcription in PHA-stimulated peripheral blood lymphocytes (36). Nuclear extracts from PHA-stimulated PBMCs were analyzed by mobility shift assays with GB1 and GB2 probes, and the consensus Ikaros binding site was derived from the mouse CD3 δ-chain gene enhancer (42). These results revealed that dexamethasone inhibited the binding of nuclear transcription factors to the Ikaros GB1 site (Fig. 3A) as well as to the consensus Ikaros binding site (Fig. 3B, IK) and reduced AP-1 binding activity, whereas CBF binding activity to the GB2 site remained unaffected (Fig. 4).

Both Ikaros and AP-1 Binding Sites Participate in Dexamethasone-mediated Inhibition of Granzyme B Promoter Activity—Two binding sites, the Ikaros and AP-1/CBF binding sites, have been demonstrated to be essential for granzyme B promoter activation (36–38). To further evaluate the contribution of these sites to the inhibitory effect of dexamethasone on the granzyme B promoter, we introduced mutations able to selectively abolish binding of complexes to the Ikaros, AP-1, or AP-1/CBF sites in the context of the granzyme B promoter (36). To gain sensibility in this analysis, these granzyme B promoter constructs were introduced in a luciferase promoterless reporter construct. As shown in Fig. 5A, the transcriptional activity of the granzyme B promoter in PHA-stimulated PBMCs was significantly inhibited by dexamethasone. Mutations that abolished the binding of the nuclear factors on the Ikaros binding site partially reduced the granzyme B promoter activity in PHA-activated cells, but treatment with dexamethasone was still able to modulate the residual luciferase activity driven by the Ikaros-mutated promoter fragment. This result is in favor of a role for the AP-1 binding site in dexamethasone-mediated inhibition. Mutations of the AP-1/CBF binding site or selective mutations of the AP-1 binding site also reduced the level of granzyme B promoter activity in PHA-activated cells. An inhibitory effect of dexamethasone on the mutated promoter containing an intact Ikaros binding site was still observed, confirming the role of the Ikaros regulatory element in dexamethasone-mediated inhibition. Similar results were obtained by insertion of a limited region of the granzyme B promoter (−148 to −77), which only contains wild type or mutated Ikaros and AP-1/CBF binding sites upstream of the SV40 promoter in the pGL3 vector (data not shown).

Altogether these data show that the two DNA binding sites, Ikaros and AP-1, both represent critical regulatory targets for dexamethasone-induced down-regulation of the granzyme B promoter.

Considering the critical role of the AP-1 binding site, we tested the effect of cotransfection of a c-Jun expression vector on the dexamethasone-mediated inhibition of the granzyme B promoter. As shown in Fig. 5B, cotransfection of c-Jun expression vector partially restores PHA activation of the granzyme B promoter in presence of dexamethasone. In addition, the cotransfection experiment with c-Jun cannot reverse glucocorticoid-mediated repression of CAT2AM2 (77), corresponding to mutation on the AP-1/CBF binding site with an intact Ikaros binding site but is very potent at reversing repression of CAT2AM1 (77), corresponding to the mutation in the Ikaros binding site with an intact AP-1/CBF binding site (Fig. 5C). These results show that c-Jun cotransfection has no effect on the Ikaros binding site activity. The Dex treatment and the c-Jun cotransfection have no effect on the PBLCAT2 vector activity (data not shown).

As potential candidate proteins that could interact with the granzyme B Ikaros regulatory element, we studied the effect of dexamethasone on Ikaros protein expression in PHA-activated PBMCs. Western blot analysis of nuclear extracts revealed that dexamethasone treatment is associated with a moderate decrease of the bands whose size correspond to Ik1 and Ik2/3 isoforms (45, 47, 48) and appearance of a band with a smaller
size (Fig. 6). In contrast, the expression of Ikaros proteins was unchanged by the dexamethasone treatment in whole extracts (data not shown). These results suggested that dexamethasone modulates protein nuclear expression of Ikaros isoforms.

However, cotransfection experiments with Ik1 and Ik2 expression vectors (47) did not restore activity of the granzyme B promoter in dexamethasone-treated PHA PBMCs (data not shown), suggesting that overexpression of these isoforms per se is not able to counterbalance the inhibitory effect of dexamethasone.

DISCUSSION

Although glucocorticoids are among the most potent and widely used immunosuppressive agents, the mechanisms by which they suppress the immune response have not yet been clearly defined. Glucocorticoids have been shown to be able to affect monocyte and T lymphocyte proliferation and function (49–51). The main mechanism by which they exert their powerful effects is modulation of the expression of specific sets of genes (49). Glucocorticoids have been shown to inhibit cytokine expression at both the transcriptional and post-transcriptional levels. Recently, in addition to its distal effect on gene expression, dexamethasone has been described as down-regulating T-cell activation by inhibiting the transduction of activation signals initiated upon T-cell receptor occupancy (52) and IL-2 receptor (53, 54).

In contrast to IL-2 and interferon γ gene expression, which are early events in T-cell activation, induction of granzyme B gene transcription is a late-phase event that characterizes the acquisition of the cytotoxic function by T lymphocytes. The results of this study demonstrated that inhibition of late-phase granzyme B gene expression by glucocorticoids occurred at the transcriptional level, resulting in a concentration-dependent inhibition of granzyme B gene promoter activity.

Glucocorticoids exert their effects after diffusing into target cells, where they bind to their cytoplasmic receptor. This complex then translocates to the nucleus, where it inhibits the transcription of target genes. This inhibition can occur through direct binding to the glucocorticoid response elements, located within the promoter region of the genes (55). No differential DNA binding activity to putative glucocorticoid receptor element sites identified within the granzyme B promoter by sequence homologies to glucocorticoid receptor element consensus sequences could be detected (data not shown).

Other mechanisms of glucocorticoid suppressive effects that
Ikaros binding site activity of the granzyme B promoter. PHA-activated activity in the presence of dexamethasone but had no effect on the less vector.

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S.E. of three individual experiments in duplicate. CAT3 is a promoter- absence of dexamethasone (taken as 100%) represents the mean value

The percentage of activation relative to the CAT3A activity in the experiment was performed three times in duplicate with similar results

were activated with PHA at a final concentration of 15

human granzyme B activity in presence of dexamethasone. PBMCs

induced down-regulation of the granzyme B promoter. PHA-activated

culture, 107 cells were cotransfected with 10

promoter constructs AM1 (mutated Ikaros binding site), AM2 (AP-1/CBF

of the wild type A(77) −148 to −77 granzyme B promoter and mutated promoter construct AM1(77) (mutated Ikaros binding site), AM2(77)

DNA-binding Ikaros isoforms (Ik1, 2, and 3) greatly increases their affinity for DNA and their consequent ability to activate transcription. Heterodimers formed between isoforms Ik1, 2, and 3 with Ik4, 5, and 6 lead to transcriptionally inactive forms (61). Hence, heterodimers that include one Ikaros isoform that lacks a DNA binding domain are transcriptionally inert. The observation of a new band detected by Western blot using the anti-Ikaros antibody with nuclear extracts upon dexamethasone treatment is compatible with the expression of such an isoform lacking the DNA binding domain. As recently suggested, such isoforms could interfere with the activity of Ikaros or Ikaros-like binding factors such as Aiolos, Helios, p30 (62, 63), or other yet-undefined partners in a dominant negative

involve antagonism of the action of transcription factors required for transcriptional activation have been described (55). Inhibition of IL-2 gene transcription is thought to be the consequence of negative interference with AP-1 and NFAT (nuclear factor of activated T-cells) nuclear transcriptional factors, which have been previously demonstrated to be of crucial importance for the activity of this cytokine promoter (18, 19). Although AP-1 is involved in lymphokine gene induction, interference with AP-1 activity cannot account for the full spectrum of immunoregulatory genes affected by glucocorticoids. More recently, glucocorticoids have been shown to inhibit the activity of the NF-κB transcription factor by increasing transcription and protein synthesis of its cytoplasmic inhibitor IκBa (27, 29). Previous studies have shown that dexamethasone represses the DNA binding activity of nuclear NF-κB (28, 30). Glucocorticoid suppression of a member of the rat IL-8 family is also mediated by impairment of NF-κB activation (24).

In this report, we demonstrate that dexamethasone inhibits granzyme B gene promoter activity induction by interfering with the binding of nuclear transcription factors to the Ikaros GB1 site and by reducing the amount of AP-1 binding activity while not affecting CBF binding activity to the GB2 site.

The mechanism by which AP-1 is inhibited by glucocorticoids is well documented and implicates interaction between the glucocorticoid receptor and Jun and Fos, the two proteins that transactivate transcription when binding to the AP1 site (28, 56–59). In fact, in our model, Fos protein nuclear expression is not down-regulated by dexamethasone (data not shown).

The role of an Ikaros binding site in the dexamethasone-mediated inhibition of gene transcription has not yet been described. Candidate proteins that could bind the granzyme B Ikaros site are the different isoforms (Ik1, 2, 3, 4, 5, 6) encoded by the Ikaros gene (45, 47, 48), an essential regulator of lymphocyte differentiation (42, 60). Ikaros activity is controlled by the nature of the complexes formed between its expressed isoforms (61). Homo- and heterodimer formation between the DNA-binding Ikaros isoforms (Ik1, 2, and 3) greatly increases their affinity for DNA and their consequent ability to activate transcription. Heterodimers formed between isoforms Ik1, 2, and 3 with Ik4, 5, and 6 lead to transcriptionally inactive forms (61). Hence, heterodimers that include one Ikaros isoform that lacks a DNA binding domain are transcriptionally inert. The observation of a new band detected by Western blot using the anti-Ikaros antibody with nuclear extracts upon dexamethasone treatment is compatible with the expression of such an isoform lacking the DNA binding domain. As recently suggested, such isoforms could interfere with the activity of Ikaros or Ikaros-like binding factors such as Aiolos, Helios, p30 (62, 63), or other yet-undefined partners in a dominant negative

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fashion. Dexamethasone treatment could generate the presence of the nucleus of a dominant negative isoform. This interpretation is compatible with the absence of retarded bound complexes in bandshift assay. Moreover, our preliminary results are not in favor of an enhancement of granzyme B promoter activity consequent to independent cotransfection of recombinant Ik1 and Ik2 isoforms. This suggests that other lineage-specific partners may interact with the granzyme B Ikaros regulatory element. Further analyses are necessary to elucidate dexamethasone inhibition mechanisms involving the Ikaros regulatory element.

In conclusion, we have demonstrated that Ikaros and AP-1 binding sites of the human granzyme B promoter are both critical for the dexamethasone-induced down-regulation of the granzyme B gene expression.
Down-regulation of Human Granzyme B Expression by Glucocorticoids: DEXAMETHASONE INHIBITS BINDING TO THE Ikaros AND AP-1 REGULATORY ELEMENTS OF THE GRANZYME B PROMOTER
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