Aims: Nitric oxide (NO) has been increasingly implicated in inflammatory skin diseases, namely in allergic contact dermatitis. In this work, we investigated the effect of dexamethasone on NO production induced by the epidermal cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) in a mouse fetal skin dendritic cell line. **Methods:** NO production was assessed by the method of Griess. Expression of the inducible isoform of nitric oxide synthase (iNOS) protein was evaluated by western blot analysis and immunofluorescence microscopy. Western blot analysis was also performed to evaluate cytosolic IkappaB-alpha (IkB- α) protein levels. The electrophoretic mobility shift assay was used to evaluate the activation or inhibition of nuclear factor kappa B (NF- κ B). **Results:** GM-CSF induced iNOS expression and NO production, and activated the transcription factor NF- κ B. Dexamethasone inhibited, in a dose-dependent manner, NO production induced by GM-CSF. Addition of dexamethasone to the culture, 30 min before GM-CSF stimulation, significantly inhibited the cellular expression of iNOS. Dexamethasone also inhibited GM-CSF-induced NF- κ B activation by preventing a significant decrease on the IkB- α protein levels, thus blocking NF- κ B migration to the nucleus. **Conclusions:** The corticosteroid dexamethasone inhibits GM-CSF-induced NF- κ B activation, iNOS protein expression and NO production. These results suggest that dexamethasone is a potent inhibitor of intracellular events that are involved on NO synthesis, in skin dendritic cells.

Key words: Granulocyte–macrophage colony-stimulating factor, Nitric oxide, Transcription factors, Dexamethasone, Skin dendritic cell

Dexamethasone prevents granulocyte–macrophage colony-stimulating factor-induced nuclear factor- κ B activation, inducible nitric oxide synthase expression and nitric oxide production in a skin dendritic cell line

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Introduction

Nitric oxide (NO), synthesized in large quantities by the inducible isoform of NO synthase (iNOS), has a dual role in human health, behaving as a ‘double edge sword’. This free radical acts as a cytotoxic agent against exogenous microorganisms, by stimulating macrophage-induced inflammation and death of infected cells. On the other hand, it favors exaggerated tissue injury in sepsis, hypersensitivity or autoimmunity, by promoting inflammation or cytotoxicity of innocent bystander cells.¹ In the skin, NO is produced by iNOS in several cells, namely in keratinocytes, fibroblasts, Langerhans cells and other dendritic cells. Apart from its potential beneficial activity against external aggressors, it is increasingly reported to be involved in skin inflammatory and immune responses,^{2,3} namely contact dermatitis,^{4,5} both allergic contact dermatitis^{6,7} and irritant contact dermatitis,^{6,8} atopic dermatitis,^{9,10} and psoriasis.^{11–13} Among these diseases, which show a good clinical response to topical glucocorticosteroids

(GCs),^{14,15} a topical iNOS inhibitor has also been shown to be effective in atopic dermatitis¹⁰ and psoriasis.¹³

After stimulation by microorganisms and epidermal cytokines, like interferon- γ , tumor necrosis factor- α , interleukin 1- β and granulocyte–macrophage colony-stimulating factor (GM-CSF), Langerhans and other dendritic skin antigen presenting cells produce NO,^{16–18} which may be important in the process of antigen presentation to T cells.^{19,20} We have previously shown, using a mouse fetal skin dendritic cell line (FSDC) in culture, that GM-CSF, a cytokine produced by allergen-stimulated keratinocytes that promotes Langerhans cell maturation and antigen presentation, induces NO production.²¹ This is dependent on the expression of iNOS and is associated with the activation of the transcription nuclear factor kappa B (NF- κ B).²² This transcription factor is present in a latent (inactive) state in the cytoplasm, bound to an inhibitor, IkappaB (IkB).²³ Exposure of cells to a variety of physiological and non-physiological stimuli induces phosphorylation, and subse-

quent degradation, of I κ B protein.²⁴ This process releases NF- κ B from this inhibitor, enabling it to translocate to the nucleus,²⁵ where it increases the expression of specific genes, including those involved in iNOS expression.^{26,27}

Glucocorticoids, which exert a multifunctional anti-inflammatory activity in several skin diseases and affect Langerhans cells maturation,²⁸ have been shown, both *in vivo* and *in vitro*, to interfere with the activity of iNOS and NF- κ B in some pathological processes and in some cell lines.^{29–31} In this work, we investigated the effect of dexamethasone on a FSDC cell line stimulated by GM-CSF, evaluating NO production, iNOS expression and NF- κ B activation. Our results showed that dexamethasone prevents GM-CSF-induced reduction of the cytosolic levels of the NF- κ B inhibitor, I κ B- α , and the subsequent translocation of the NF- κ B proteins into the nucleus, where it is likely to mediate the observed inhibition of iNOS expression and NO production.

Materials and methods

Materials

The rabbit anti-mouse iNOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY, USA) and the rabbit anti-I κ B- α polyclonal antibody from New England BioLabs Inc. (Beverly, MA, USA). The NF- κ B consensus oligonucleotide was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), whereas the mouse monoclonal antibody against actin and the protease inhibitor cocktail were obtained from Roche (Carnaxide, Portugal). The fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin was from DAKO (Copenhagen, Denmark). The mounting medium for fluorescence, Vectashield, was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). The ³²P-labeled γ -ATP, T4 polynucleotide kinase, poly(dI–dC).poly(dI–dC), the ECL western blotting analysis system, the horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin and the X-ray films were from Amersham Biosciences (Carnaxide, Portugal). The horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was from Pierce (Rockford, IL, USA). The mouse rGM-CSF was from R&D systems (Minneapolis, MN, USA), fetal calf serum was from Biochrom KG (Berlin, Germany) and trypsin was from Invitrogen (Paisley, UK). Dexamethasone was obtained from Sigma-Aldrich Química (Sintra, Portugal). Triton[®]X 100 and paraformaldehyde were from VWR International/Merck Eurolab (Lisboa, Portugal). All other reagents were from Sigma Chemical Co (St Louis, MO, USA).

Cell culture

The fetal mouse skin dendritic cell line FSDC, kindly supplied by Dr G. Girolomoni (Laboratory of Immunology, Istituto Dermopatico dell'Immacolata, IRCCS, Rome, Italy), is a skin dendritic cell precursor with antigen presenting capacity.³² The cells were cultured in endotoxin free Iscove's Modified Dulbecco's Medium, supplemented with 10% (v/v) fetal calf serum, 1% (w/v) glutamine, 3.02 g/l of sodium bicarbonate, 100 μ g/ml of streptomycin and 100 U/ml of penicillin. For western blot and electrophoretic mobility shift assay (EMSA) analysis, FSDC were plated at 2×10^6 cells/well, in six-well culture plates, whereas for nitrite measurements the cells were plated at 0.2×10^6 cells/well, in 48-well culture plates. For immunofluorescence analysis, FSDC cells were grown on Lab-Tek chamber slides with a cover (0.2×10^6 cells/slide).

Nitrite measurement

The production of NO was accessed as the accumulation of nitrite (NO₂[−]) in the culture supernatants, using a colorimetric reaction with the Griess reagent, as described previously.²² Briefly, cells were stimulated with GM-CSF (100 ng/ml) for 48 h, alone (control) or in the presence of increasing concentrations of dexamethasone (0.0025–1 μ M), added to the cell culture 30 min before GM-CSF. In another set of experiments, the cells were incubated for 48 h with the same concentrations of dexamethasone, in the absence of other stimuli.

At 48 h, culture supernatants were collected and diluted with equal volumes of the Griess reagent (0.1% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% (w/v) sulfanilamide and 5% (w/v) H₃PO₄). After 10 min incubation, the absorbance was measured at 550 nm in an automated plate reader (SLT Spectra; Salzburg, Austria). The nitrite concentration was determined from a sodium nitrite standard curve.

Western blot analysis

For immunodetection of iNOS, cells were treated with culture medium alone (control) or with GM-CSF (100 ng/ml) for 24 h, in the absence or in the presence of the corticosteroid dexamethasone (0.5 μ M), added to the cell culture 30 min before the GM-CSF. As a second control, cells were also treated with dexamethasone (0.5 μ M) alone for the same time period. After treatment, cells were washed twice with phosphate-buffered saline (PBS) and total cell lysates were obtained after harvesting the cells in a sonication buffer containing 0.32 M sucrose, 10 mM Tris–HCl (pH 7.5), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol, 0.1 mM phenylmethyl-

sulfonylfluoride and the protease inhibitor cocktail. Then, the lysates were incubated on ice for 30 min and sonicated to disrupt the cells. The protein concentration was determined using the bicinchoninic acid method.

For immunodetection of I κ B- α , FSDC cells were maintained in the culture medium alone (control) or were treated with GM-CSF (100 ng/ml) for 15, 45 and 60 min. To evaluate the effect of dexamethasone, cells were stimulated for 45 min with GM-CSF (100 ng/ml) after pre-incubation with dexamethasone (0.5 μ M), for 30 min. Proteins of the cytosolic fraction were obtained after harvesting the cells in 10 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) Nonidet P-40, 1 mM dithiothreitol, 10 mM Tris-HCl (pH 7.5), 0.1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail. The lysates were incubated on ice for 15 min, and the cytosolic proteins were isolated from the supernatant obtained after centrifugation at 2300 $\times$ g for 10 min. The supernatant containing the cytosolic proteins was collected, and the protein concentration was determined using the bicinchoninic acid solution.

Protein samples were separated on 10% (v/v) (for iNOS detection) or 15% (v/v) (for I κ B- α detection) sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) dry milk in Tris-buffered saline with 0.1% (v/v) Tween 20 for 1 h. The levels of iNOS and I κ B- α proteins were detected using, respectively, a rabbit polyclonal anti-mouse iNOS antibody (1:2000) and a rabbit polyclonal anti-I κ B- α antibody (1:1000) for 1 h, followed by incubation with a horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:5000). The immunocomplexes were visualized by the enhanced chemiluminescence (ECL) method. To demonstrate equivalent protein loading, the membranes were stripped and reprobed with an anti-actin antibody.

The blots were digitally generated using a HP ScanJet 5p and processed in the Corel Photo-Paint program.

Immunofluorescence microscopy

For immunofluorescence analysis, FSDC cells were treated with culture medium alone (control) or with GM-CSF (100 ng/ml) for 24 h, in the presence or in the absence of the corticosteroid, dexamethasone (0.5 μ M), added to the cell culture 30 min before the GM-CSF. The cells were then washed with PBS and fixed with PBS containing 4% (w/v) sucrose and 4% (w/v) paraformaldehyde for 15 min. FSDC were then permeabilized in PBS containing 1% (v/v) Triton[®]X 100 for 10 min. Non-specific binding was blocked by incubation of the cells with PBS supplemented with normal swine serum (1:20) and 0.5% (w/v) bovine

serum albumin (BSA) for 45 min at room temperature. Cells were then incubated for 90 min at room temperature, with a rabbit polyclonal antibody directed against mouse iNOS (10 μ g/ml). After rinsing with PBS the cells were incubated with FITC-conjugated swine anti-rabbit immunoglobulin (1:40) in 0.5% BSA-PBS for 45 min. The cells were rinsed again and mounted with the mounting medium for fluorescence Vectashield. Cells labeled with FITC-anti-iNOS were photographed on a Zeiss Axioskop microscope. Control experiments consisted of processing the same preparations as described, except for the omission of the primary antibody, and resulted in no specific staining.

Electrophoretic mobility shift assay

FSDC cells were kept in the culture medium alone (control), were incubated with dexamethasone (0.5 μ M) or were treated with GM-CSF (100 ng/ml) for 15, 45 and 60 min. In another set of experiments, cells stimulated for 45 min with GM-CSF (100 ng/ml) were also incubated with dexamethasone (0.5 μ M), added to the culture 30 min before the GM-CSF. The cells were then washed with PBS and lysed in 10 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) Nonidet P-40, 1 mM dithiothreitol, 10 mM Tris-HCl (pH 7.5), 0.1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail. The lysates were incubated on ice for 15 min and centrifuged at 2300 $\times$ g for 10 min. The pellet obtained was resuspended in 300 mM NaCl, 5 mM MgCl₂, 20% (v/v) glycerol, 1 mM dithiothreitol, 0.2 mM EDTA, 20 mM HEPES buffer (pH 7.5), 0.1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail, incubated on ice for 1 h and centrifuged at 12,000 $\times$ g for 20 min. The supernatant containing the nuclear proteins was collected, and the protein concentration was determined using the bicinchoninic acid method.

The EMSA method used was similar to that described previously³³ with slight modifications. The probes consisted of a double stranded oligonucleotide containing the consensus binding sequence for NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3') end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase. Typical binding reactions consisted of 12 μ g of nuclear extract, about 100,000 cpm of [γ -³²P]-labeled oligonucleotide and 100 μ g/ml of poly(dI-dC).poly(dI-dC) in a buffer containing 20 mM HEPES (pH 7.9), 1 mM MgCl₂, 4% (w/v) Ficoll 400, 0.5 mM dithiothreitol, 50 mM KCl, and 1 mg/ml of BSA, and were incubated at room temperature for 45 min. Binding reactions were separated on 7% (v/v) non-denaturing polyacrylamide gels, in a buffer system containing 0.044 M Tris-Base (pH 8.0), 4.45 mM boric acid and 1 mM EDTA, at a constant voltage of 150 V, for 2h 30 min at room temperature. The gels

were transferred to Whatman paper, dried, and subjected to autoradiography.

In competition experiments, an excess of unlabeled oligonucleotide ($100\times$) was added to the nuclear extracts for 30 min before addition of radiolabeled probe.

The blots were digitally generated using a HP ScanJet 5p and processed in the Corel Photo-Paint program.

Data analysis

Results are presented as mean $\pm$ standard error of the mean of the indicated number of experiments. Mean values were compared using one-way analysis of variance and the Bonferroni's multiple comparison test. The significance levels were $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

Dexamethasone inhibits NO production in GM-CSF-stimulated FSDC in a dose-dependent manner

We have previously reported that GM-CSF causes a dose-dependent increase in nitrite concentration in culture supernatants, reflecting an increase in NO production by the FSDC.²² In the present work, 100 ng/ml of GM-CSF was used, which was previously shown to increase significantly NO production.²²

Cell stimulation with GM-CSF for 48 h significantly increased nitrite production, from $2.7 \pm 1.1 \mu\text{M}$ when FSDC were incubated with culture medium alone to $4.2 \pm 1.5 \mu\text{M}$ when FSDC were incubated with 100 ng/ml of GM-CSF. To investigate the effect of the dose of dexamethasone on GM-CSF-induced NO production, the corticosteroid was added in increasing concentrations (0.0025–1 μM) to the culture, 30 min before stimulation with GM-CSF. Dexamethasone inhibited nitrite production induced by GM-CSF in a dose-dependent manner (Fig. 1), with an IC_{50} value of $3.2 \times 10^{-3} \mu\text{M}$, as calculated by the Hill plot. A concentration of 0.5 μM dexamethasone, which completely inhibited NO production, was used in subsequent experiments to study its mechanism of action. Dexamethasone, in the absence of GM-CSF stimulation, had no effect on NO production (data not shown).

Cellular viability was evaluated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and by the Trypan blue assay, as described previously.²¹ The results indicated that there was no significant toxicity of dexamethasone in the concentrations used (data not shown).

FIG. 1. Dose-dependent effect of dexamethasone on GM-CSF-induced nitrite production in FSDC. FSDC (0.2×10^6 cells) were pre-treated for 30 min with a range of concentrations of dexamethasone (0.0025–1 μM), as indicated, and then stimulated with 100 ng/ml GM-CSF for 48 h. Results were expressed as percentage of maximal nitrite production on the supernatants of cells maintained in culture medium, in the presence of GM-CSF and in the absence of dexamethasone. Nitrite levels in the culture supernatants were detected by the Griess reaction as described in Materials and methods. Each value represents the mean $\pm$ standard error of the mean from five experiments.

Dexamethasone inhibits iNOS protein expression in FSDC stimulated with GM-CSF

Western blot analysis was used to characterize iNOS protein levels in non-treated cells (control), in cells treated with GM-CSF, in the presence or in the absence of dexamethasone, and in cells treated only with dexamethasone. As indicated in Fig. 2A,B, control cells (i.e. non-activated cells) expressed a residual level of iNOS protein (130 kDa) (lane 1). In cells stimulated with GM-CSF, the expression of iNOS protein was increased by 106% relative to control cells (lane 2). In GM-CSF-stimulated cells treated with dexamethasone, the expression of iNOS protein was significantly inhibited, decreasing to 42% of the GM-CSF-stimulated cell levels (lane 4) and to 85% of the iNOS levels expressed by control cells. When the effect of dexamethasone was tested alone, we observed a reduction of iNOS expression to 68% of the control, which did not reach statistical significance (lane 3). This decrease in iNOS protein expression correlated well with the inhibitory effect of dexamethasone on NO production, as shown in Fig. 1.

Immunofluorescence assay was performed to confirm these results, under the same conditions used for western blot. Fig. 2C shows that control cells (i.e. cells maintained in culture medium) expressed a residual level of iNOS protein. In cells treated with GM-CSF, the fluorescence was markedly increased, showing a significant increase in iNOS protein expression. GM-CSF-stimulated cells treated with dexamethasone exhibited a similar, although weaker, fluorescence than control. The fluorescence exhib-

FIG. 2. Effect of dexamethasone (Dex) on GM-CSF-induced iNOS protein expression in FSDC. (A) Western blot analysis of iNOS and actin protein levels. FSDC cells (2×10^6 cells) were incubated for 24 h with culture medium alone (control, lane 1), with 100 ng/ml of GM-CSF (lane 2), with 100 ng/ml of GM-CSF in the presence of 0.5 μ M dexamethasone (lane 4), or with 0.5 μ M dexamethasone alone (lane 3). When the effects of dexamethasone on the response to GM-CSF was investigated, the cells were pre-incubated with the corticosteroid for 30 min. Total cell extracts were electrophoresed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and subjected to western blot analysis using an anti-iNOS antibody as described in Materials and methods. The membrane was stripped and reprobed with anti-actin antibody to confirm equal protein loading. The blot shown is representative of three blots yielding similar results. (B) The results obtained in three independent experiments were quantified with an image analyzer and the results are presented as means $\pm$ standard error of the mean. *** $p < 0.001$, as determined by one-way analysis of variance with Bonferroni's multiple comparison test. (C) Immunofluorescence analysis of iNOS protein. FSDC cells (0.2×10^6) were incubated under the same conditions as performed for western blot assay.

ited by cells treated only with dexamethasone was minimal, the weakest of all. All these results correlated well with the results obtained in the western blot assays (Fig. 2A).

Dexamethasone blocks the activation of NF- κ B in FSDC stimulated with GM-CSF

The effect of dexamethasone on NF- κ B activation induced by GM-CSF in FSDC cells was evaluated in nuclear extracts by EMSA analysis. The cells were

incubated with GM-CSF for 15, 45 and 60 min to determine the effect of cytokine on the activation of NF- κ B, or were pre-treated with dexamethasone for 30 min and then stimulated with GM-CSF for 45 min in order to investigate the effect of the corticosteroid. As shown in Fig. 3A,B, GM-CSF induced NF- κ B binding to DNA, exhibiting a maximal effect at 45 min when the NF- κ B binding activity was increased by 101% relative to control cells (lanes 2 and 4). Pre-treatment with dexamethasone prevented NF- κ B binding to DNA in GM-CSF-stimulated cells. Fig.

FIG. 3. Effect of dexamethasone (Dex) on GM-CSF-induced NF- κ B binding to DNA in FSDC. FSDC (2×10^6 cells) were incubated in culture medium alone (control, lane 2) or in the presence of 100 ng/ml of GM-CSF, for 15, 45 and 60 min (lanes 3–5). In lane 6 the cells were pre-incubated with 0.5 μ M dexamethasone for 30 min before stimulation with GM-CSF and dexamethasone for 45 min. Control experiments, where the cells were incubated with dexamethasone for 75 min, are shown in lane 7. Unlabeled oligonucleotide (i.e. non-radioactive NF- κ B consensus containing oligonucleotide) was used in excess (100-fold) to demonstrate specificity of the induced bands (lane 8). (A) Nuclear extracts were subjected to EMSA analysis as described in Materials and methods. The blot shown is representative of three blots yielding similar results. (B) The bands were quantified with an image analyzer and the results are shown as means $\pm$ standard error of the mean from three independent experiments. ** $p < 0.01$ and *** $p < 0.001$, as determined by one-way analysis of variance with Bonferroni's multiple comparison test.

3A,B shows that pre-incubation of the cells with dexamethasone for 30 min before stimulation with GM-CSF for 45 min, in the presence of the corticosteroid, fully blocked GM-CSF-induced NF- κ B binding to DNA (lane 6). Under these experimental conditions, NF- κ B binding to DNA was decreased to 55% of the activity found in GM-CSF-stimulated cells and was not significantly different from the control. Dexamethasone alone had no effect on NF- κ B binding to DNA (10% of difference from the control) (lane 7).

As control for the gel shift assay, unlabeled oligonucleotide (100-fold excess) was used (lane 8), which inhibited the NF- κ B complex formation.

Dexamethasone inhibition of NF- κ B in GM-CSF-stimulated FSDC is mediated by modulation of cytosolic I κ B- α levels

As we have previously shown, GM-CSF induces an I κ B- α degradation in FSDC.²² To determine whether

the suppressive effect of dexamethasone on GM-CSF-induced NF- κ B activation was due to the modulation of its inhibitor, I κ B- α , the levels of this protein on cytosolic cell extracts were examined by western blot analysis.

As shown in Fig. 4A,B, we observed that GM-CSF induced a maximal reduction in I κ B- α protein levels at 45 min, when a reduction in the protein levels to 44% of the control was observed (lanes 1 and 3). This reduction was fully inhibited when FSDC were pre-incubated with dexamethasone for 30 min and then stimulated with GM-CSF for 45 min in the presence of the corticosteroid (lane 5), where an increase of 93% in I κ B- α protein levels was observed comparative with GM-CSF-stimulated cells in the absence of dexamethasone. Indeed, the amount of I κ B- α present in FSDC stimulated with GM-CSF in the presence of dexamethasone was not significantly different from the protein levels in control cells. These results show that there is a correlation between the inhibitory

FIG. 4. Effect of dexamethasone (Dex) on GM-CSF-stimulated IκB-α decrease in FSDC. FSDC (2×10^6 cells) were incubated in culture medium alone (control, lane 1) or in the presence of 100 ng/ml of GM-CSF for 15, 45 and 60 min (lanes 2–4). Lane 5 corresponds to cells pre-incubated with 0.5 μM dexamethasone for 30 min before the stimulation with 100 ng/ml of GM-CSF for 45 min. Cytosolic cell extracts were electrophoresed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and subjected to immunoblot analysis using an anti-IκB-α antibody as described in Materials and methods. The membrane was stripped and reprobed with an anti-actin antibody to confirm equal protein loading. (A) Western blot analysis of IκB-α and actin protein levels. The blot shown is representative of three blots yielding similar results. (B) The bands were quantified with an image analyzer and the results are shown as means $\pm$ standard error of the mean from three independent experiments. *** $p < 0.001$, as determined by one-way analysis of variance with Bonferroni's multiple comparison test.

effect of dexamethasone on GM-CSF-induced NF- κ B activation and on GM-CSF-induced IκB-α protein down-regulation.

Discussion

The anti-inflammatory properties of GCs such as dexamethasone are due to the down-regulation of the expression of immunomodulatory genes,^{26,30} thereby inhibiting the expression of enzymes involved in the inflammatory process, namely iNOS, which produces NO in large amounts.^{29,34} GCs mediate these effects through the intracellular glucocorticoid receptor and, depending on the target gene, the complex ligand–glucocorticoid receptor may stimulate (transactivation), or alternatively inhibit (transrepression), gene transcription.^{30,35} In this

work, we show that, in FSDC, the glucocorticoid dexamethasone inhibits iNOS protein expression and nitrite production induced by GM-CSF. Furthermore, dexamethasone also prevents the decrease of IκB-α protein levels, and subsequent NF- κ B translocation to the nucleus, induced by GM-CSF (Fig. 3 and Fig. 4). NO has previously been involved in skin inflammatory processes, namely in psoriasis,¹¹ allergic contact dermatitis,^{6,7} atopic dermatitis,⁹ and other inflammatory skin diseases,² where topical GCs have shown to be beneficial.

GM-CSF is a multipotent cytokine that mediates the development of immunologically active DC in culture^{36–38} and that is involved in inflammatory processes.³⁷ In the skin, the epidermal cytokine GM-CSF is produced by keratinocytes upon stimulation by allergens and it promotes Langerhans cell maturation, thus favoring antigen presentation to T cells.³⁹ Allergic contact dermatitis has an inflammatory component, and therefore GM-CSF is probably involved in this pathology.

In our previous work, we observed that, in FSDC, GM-CSF induces iNOS protein expression and NO production in a process dependent on the nuclear migration of NF- κ B, which occurs upon phosphorylation and degradation of their cytosolic inhibitor, IκB-α.²² IκB-α protein is a member of a family of inhibitory molecules that masks the nuclear localization sequence of NF- κ B, inhibiting its binding to DNA.^{24,25,40,41} Here, we demonstrated that, in FSDC cells, dexamethasone prevents the decrease of cytosolic IκB-α protein levels induced by GM-CSF (Fig. 4), and inhibits NF- κ B binding to DNA (Fig. 3). In contrast, activated NF- κ B may then bind to the iNOS gene promoter region, thereby stimulating iNOS protein expression. Accordingly, the promoter region of the iNOS gene contains binding sites for the NF- κ B.²³

The induction of allergic responses by chemical sensitizers encountered on the skin is dependent upon the action of cytokines produced locally.³⁷ These skin cytokines may also serve to influence the inflammatory and immune responses provoked by chemical sensitizers and, concomitantly, the characteristics of allergic diseases.³⁷ The anti-inflammatory properties of glucocorticoids on iNOS expression and NO production induced by the epidermal cytokine GM-CSF had not been described before. The results presented here show that the inhibitory effect of dexamethasone on GM-CSF-induced iNOS expression and NO production can account for the anti-inflammatory effect of this glucocorticoid.

A better knowledge of the signaling pathways activated in DC that induce iNOS expression and the mechanism of action of drugs, like GCs, useful in the treatment of cutaneous inflammatory processes,

can open a window to our understanding of these diseases and their treatment.

ACKNOWLEDGEMENT. This work was supported by the Portuguese Foundation for Science and Technology, project number 36373/2000.

References

- Bruch-Gerharz D, Ruzicka T, Kolb-Bachofen V. Nitric oxide in human skin: current status and future prospects. *J Invest Dermatol* 1998; **110**: 1–7.
- Rowe A, Farrell AM, Bunker CB. Constitutive endothelial and inducible nitric oxide synthase in inflammatory dermatoses. *Br J Dermatol* 1997; **136**: 18–23.
- Weller R. Nitric oxide — a newly discovered chemical transmitter in human skin. *Br J Dermatol* 1997; **137**: 665–672.
- Ross R, Gillitzer C, Kleinz R, et al. Involvement of NO in contact hypersensitivity. *Int Immunol* 1998; **10**: 61–69.
- Ross R, Reske-Kunz AB. The role of NO in contact hypersensitivity. *Int Immunopharmacol* 2001; **1**: 1469–1478.
- Ormerod AD, Dwyer CM, Reid A, Copeland P, Thompson WD. Inducible nitric oxide synthase demonstrated in allergic and irritant contact dermatitis. *Acta Derm Venereol* 1997; **77**: 436–440.
- Sahin S, Önder M, Sancak B, Bukan N, Güler MA. The role of nitric oxide in allergic contact dermatitis. *Arch Dermatol Res* 2001; **293**: 214–217.
- Wallengren J, Larsson B. Nitric oxide participates in prick test and irritant patch test reactions in human skin. *Arch Dermatol Res* 2001; **293**: 121–125.
- Taniuchi S, Kojima T, Hara Mt K, et al. Increased serum nitrate levels in infants with atopic dermatitis. *Allergy* 2001; **56**: 693–695.
- Morita H, Semma M, Hori M, Kitano Y. Clinical application of nitric oxide synthase inhibitor for atopic dermatitis. *Int J Dermatol* 1995; **34**: 294–295.
- Morhenn VB. Langerhans cells may trigger the psoriatic disease process via production of nitric oxide. *Immunol Today* 1997; **18**: 433–436.
- Ormerod AD, Weller R, Copeland P, et al. Detection of nitric oxide and nitric oxide synthase in psoriasis. *Arch Dermatol Res* 1998; **290**: 3–8.
- Ormerod AD, Copeland P, Shah SA. Treatment of psoriasis with topical NG-monomethyl-L-arginine, an inhibitor of NO synthesis. *Br J Dermatol* 2000; **142**: 985–990.
- Pan J, Ju D, Wang Q, et al. Dexamethasone inhibits the antigen presentation of dendritic cells in MHC class II pathway. *Immunol Lett* 2001; **76**: 153–161.
- Moser M, De Smedt T, Sornasse T, et al. Glucocorticoids down-regulate dendritic cell function *in vitro* and *in vivo*. *Eur J Immunol* 1995; **25**: 2818–2824.
- Qureshi AA, Hosoi J, Xu S, Takashima A, Granstein RD, Lerner EA. Langerhans cells express inducible nitric oxide synthase and produce nitric oxide. *J Invest Dermatol* 1996; **107**: 815–821.
- Weller R. Nitric oxide, skin growth and differentiation: more questions than answers? *Clin Exp Dermatol* 1999; **24**: 388–391.
- Lu L, Bonham CA, Chambers FG, et al. Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis. *J Immunol* 1996; **157**: 3577–3586.
- Bonham CA, Lu L, Li Y, Hoffman RA, Simmons RL, Thomson AW. Nitric oxide production by mouse bone marrow-derived dendritic cells: implications for the regulation of allogeneic T cell responses. *Transplantation* 1996; **62**: 1871–1877.
- Bonham CA, Lu L, Hoffman RA, Simmons RL, Thomson AW. Nitric oxide production by dendritic cells is associated with impairment of T cell responses. *Transplant Proc* 1997; **29**: 1116–1117.
- Cruz MT, Duarte CB, Gonçalves M, Carvalho AP, Lopes MC. Involvement of JAK2 and MAPK on type II nitric oxide synthase expression in skin-derived dendritic cells. *Am J Physiol* 1999; **277**: C1050–C1057.
- Cruz MT, Duarte CB, Gonçalves M, Figueiredo A, Carvalho AP, Lopes MC. Granulocyte-macrophage colony-stimulating factor activates the transcription of nuclear factor kappa B and induces the expression of nitric oxide synthase in a skin dendritic cell line. *Immunol Cell Biol* 2001; **79**: 590–596.
- Pahl HL. Activators and target genes of Rel/NF-kB transcription factors. *Oncogene* 1999; **18**: 6853–6866.
- Baldwin Jr AS. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996; **14**: 649–683.
- Chen FE, Ghosh G. Regulation of DNA binding by Rel/NF-kB transcription factors: structural views. *Oncogene* 1999; **18**: 6845–6852.
- Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kB pathway in the treatment of inflammation and cancer. *J Clin Invest* 2001; **107**: 135–142.
- Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kB/Rel in induction of nitric oxide synthase. *J Biol Chem* 1994; **269**: 4705–4708.
- Piemonti L, Monti P, Allavena P, et al. Glucocorticoids affect human dendritic cell differentiation and maturation. *J Immunol* 1999; **162**: 6473–6481.
- De Vera ME, Taylor BS, Wang QI, Shapiro RA, Billiar TR, Geller DA. Dexamethasone suppresses iNOS gene expression by upregulating Ikb- α and inhibiting NF-kB. *Am J Physiol* 1997; **273**: G1290–G1296.
- Almawi WY, Melemedjian OK. Negative regulation of nuclear factor-kB activation and function by glucocorticoids. *J Mol Endocrinol* 2002; **28**: 69–78.
- Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 1995; **270**: 286–290.
- Girolomoni G, Lutz MB, Pastore S, Aßmann CU, Cavani A, Ricciardi-Castagnoli P. Establishment of a cell line with features of early dendritic cell precursors from fetal mouse skin. *Eur J Immunol* 1995; **25**: 2163–2169.
- Cruz MT, Duarte CB, Gonçalves M, Carvalho AP, Lopes MC. LPS induction of I kappa B- α degradation and iNOS expression in a skin dendritic cell line is prevented by the Janus kinase 2 inhibitor, Tyrphostin B42. *Nitric Oxide* 2001; **5**: 53–61.
- Wissink S, van Heerde EC, van der Burg B, van der Saag PT. A dual mechanism mediates repression of NF-kB activity by glucocorticoids. *Mol Endocrinol* 1998; **12**: 355–363.
- Cato AC, Wade E. Molecular mechanisms of anti-inflammatory action of glucocorticoids. *Bioessays* 1996; **18**: 371–378.
- Witmer-Pack MD, Olivier W, Valinsky J, Schuler G, Steinman RM. Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J Exp Med* 1987; **166**: 1484–1498.
- Kimber I, Holliday MR, Dearman RJ. Cytokine regulation of chemical sensitization. *Toxicol Lett* 1995; **82/83**: 491–496.
- Thurnher M, Zelle-Rieser C, Ramoner R, Bartsch G, Höltl L. The disable dendritic cell. *FASEB J* 2001; **15**: 1054–1061.
- Gonçalo M. Langerhans cell in allergic contact dermatitis. *Trab Soc Port Dermatol Venereol* 1998; **56**: 79–93.
- Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 1998; **16**: 225–260.
- Cyert MS. Regulation of nuclear localization during signaling. *J Biol Chem* 2001; **276**: 20805–20808.

Received 10 December 2002

Accepted 8 January 2003