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Corticosteroid inhibits chemokines production in systemic sclerosis patients

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ABSTRACT

In this study, we evaluated glucocorticoids (GC) effects on cytokine/chemokine levels in serum samples and peripheral blood mononuclear cell (PBMC) production from systemic sclerosis (SSc) patients. We evaluated cytokine and chemokine levels in serum samples from SSc patients taking or not taking systemic glucocorticoids. PBMCs response to methylprednisolone (MP) was examined from 15 SSc patients and 8 healthy control subjects following PBMC stimulation with anti-CD3/CD28. Cytokine (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10, and IL-17A) and chemokine (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10) levels were quantified in serum and in PBMC culture supernatants by CBA or ELISA. Compared with patients not taking corticosteroids, we did not observe any significant differences in cytokines/chemokines serum levels in patients using systemic corticosteroids. After stimulation with anti-CD3/CD28, PBMCs treated with MP (100 μM), showed a significant reduction of CCL2/MCP-1 ($p = 0.001$), CCL5/RANTES ($p = 0.04$), and CXCL8/IL-8 ($p = 0.003$) levels in SSc patients. In PBMC from healthy controls, we observed decreased IFN-γ, TNF, IL-2, and IL-10 levels after MP treatment, compared with stimulated condition ($p < 0.01$ for all). However in SSc patients, we did not find any significant reduction in these cytokine levels after MP treatment. In conclusion, CCL2/MCP-1, CCL5/RANTES, and CXCL8/IL-8 are chemokines that are potentially modulated by corticosteroids *in vitro* in SSc patients, but no effect was observed on IL-2, IL-4, IL-6, IL-10, IL-17A, TFN, and IFN-γ secretion. These results suggest a potential effect of GCs on SSc treatment and may reflect the benefit of their use in some patients.

1. Introduction

Several studies indicate that systemic sclerosis (SSc) presents deregulated production of cytokines implicated in vascular damage and fibrosis. Since it has been known that Th1 cytokines, such as interferon-gamma (IFN-γ), have antifibrotic and anti-angiogenic effects, while Th2 cytokines, such as interleukin (IL)-4 and IL-13, have profibrotic and pro-angiogenic effects; SSc has often been considered a Th2 cytokine disease [1,2]. However, other mechanisms are also involved, such as an imbalance between Th17 and T regulatory cells, although their real role in SSc pathogenesis are not fully elucidated [3]. Chemokines are also involved in SSc development. In addition to their function as chemoattractants of T cells and inflammatory cells into tissues, they play a role in angiogenesis and fibrosis regulation. Some studies demonstrated, enhanced the expression of CCL2/MCP-1, CXCL8/IL-8, CXCL9/MIG, CXCL10/IL-10, and CCL5/RANTES in the serum, lung, and skin of SSc patients [4–6]. Given that the complex network of cytokines and chemokines may contribute to the pathogenesis of SSc, it is important to understand the effects of different treatments on modulation of these molecules.

Abbreviations: CBA, cytometric bead array; dcSSc, diffuse cutaneous systemic sclerosis; ELISA, enzyme-linked immunosorbent assay; GC, glucocorticoids; HC, healthy controls; IFN-γ, interferon gamma; IL, interleukin; IQR, interquartile range; lcSSc, limited cutaneous systemic sclerosis; MCP-1, monocyte chemoattractant protein-1; MIG, monokine induced by interferon gamma; MP, methylprednisolone; NSC, non stimulated cell; PBMC, peripheral blood mononuclear cell; RANTES, regulated upon activation and normal T cells expressed and secreted; SC, stimulated cell; SE, standard error; SSc, systemic sclerosis; TNF, tumoral necrosis factor

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Glucocorticoids (GC) have anti-inflammatory and immunosuppressive properties. They exert most of their biological effects through a genomic action, due to the interaction between their cytosolic receptor and the target genes, resulting in an increased expression of regulatory proteins (transactivation) or decreased production of proinflammatory proteins (transrepression) [7–9]. Previous studies demonstrated a downregulation of type 1 cytokines and subsequently increased the production of type 2 cytokines in healthy human peripheral blood mononuclear cells (PBMC) after the dexamethasone treatment [10,11]. *In vitro* studies verified that dexamethasone treatment promoted suppression of pro-inflammatory cytokine and chemokine production, such as TNF, IL-1β, IL-6, and IL-8, in healthy subjects [12,13].

Although glucocorticoids are the mainstay treatment for most rheumatic autoimmune diseases, their role in systemic sclerosis is still controversial. There is no randomized controlled study addressing the real efficacy of corticosteroids monotherapy in SSc. Despite the lack of evidence for their effectiveness, recent meta-analysis demonstrated the widespread use of glucocorticoids in SSc [14]. About 40% of SSc patients were treated with corticosteroids, with or without immunosuppressive drugs, mostly in those with the diffuse SSc [14,15]. Currently, experts share the opinion that glucocorticoids may be indicated only in patients with inflammatory myositis, synovitis, or alveolitis [16]. On the other hand, long-term, high-dose glucocorticoids have been implicated in precipitating renal crisis, a serious disease complication [17].

Corroborating this controversy about clinical use of corticosteroids in SSc treatment, experimental studies assessing *in vitro* effects of GC in PBMCs from SSc patients are lacking. Therefore, the aims of this study were to investigate the effects of current corticosteroids treatment on serum cytokine and chemokine profile of SSc patients compared to healthy controls and to determine GC effects on cytokine production from stimulated PBMCs obtained from these patients.

2. Materials and methods

2.1. Study subjects

We recruited 52 patients (49 female) for serum analysis, classified according to American College of Rheumatology criteria for SSc [18]. Patients were classified as limited cutaneous SSc (lcSSc; n = 28) or diffuse cutaneous SSc (dcSSc; n = 24) [19]. The mean disease duration, calculated from the time of onset of the first non-Raynaud phenomenon event, was 131.5 ± 122.2 months. The characteristics of SSc patients are summarized in the Table 1. From this group, we randomly selected 35 patients for serum chemokines analysis and 15 patients for PBMC culture. Eight age- and gender-matched healthy individuals served as controls for PBMC culture.

The study protocol was approved by the ethics committee of Universidade Federal de Pernambuco (CEP/CCS/UFPE 529/11), according to the principles of the Declaration of Helsinki, and informed consent was obtained from all subjects.

2.2. PBMC purification and culture

PBMCs were obtained from the heparinized blood of patients and controls. The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation (GE Healthcare Biosciences, Pittsburgh, PA, USA) method. PBMCs (1×10^6 cells/ml) were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), HEPES 10 mM (Gibco, Carlsbad, CA, USA) and penicillin (10.000 U/ml)/streptomycin (10.000 μg/ml) (Gibco, Carlsbad, CA, USA). Cells were stimulated with anti-CD3/CD28 (Ebioscience, San Diego, CA, USA) in the presence or absence of methylprednisolone 100 μM (Pfizer, New York, NY, USA). Cells were incubated at 37 °C in a humidified 5% CO_2 incubator. Culture supernatant was collected after 48 h for cytokines quantification.

2.3. Cytokine and chemokine quantification

Cytokine (IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10 and IL-17A) and chemokine (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10) levels were quantified in serum and PBMC culture supernatants. Concentrations of cytokines in culture supernatants and chemokines in serum and culture supernatants were determined by cytometric bead array (CBA), according to the manufacturer's protocol (CBA, BD Biosciences). Briefly, 50 μl serum samples were subjected to analysis in duplicate using the cytometric bead array kit on a Accuri C6 Flow Cytometer (BD, Biosciences). The concentration of serum cytokines was quantified using FCAP Array software v3.0.1. The detection limits were IFN-γ 4.1 pg/ml, TNF 3.7 pg/ml, IL-2 2.9 pg/ml, IL-4 3.3 pg/ml, IL-6 2.5 pg/ml, IL-10 3.3 pg/ml, and IL-17 4.2 pg/ml. For chemokines, the limits of detection were CXCL8/IL-8 0.2 pg/ml, CCL5/RANTES 1.0 pg/ml, CXCL9/MIG 2.5 pg/ml, CCL2/MCP-1 2.7 pg/ml, and CXCL10/IP-10 2.8 pg/ml.

Serum cytokines levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA for IL-4, IL-6, IL-10 e IFN-γ, and eBioscience, San Diego, CA, USA for IL-17, TNF-α, IL-2). The lower detection limits for the ELISA analyses were as follows: 3.9 pg/ml for IL-17 A, IL-4 and IL-10; 7.82 pg/ml for TNF; 4.69 pg/ml for IL-6; 9.37 pg/ml for IFN-γ; 1.5 pg/ml for IL-2.

2.4. Statistical analysis

Statistical analyses of the data were performed using the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) statistical program. The D'Agostino test verified the normality of samples. Numerical data were expressed as mean ± standard error (SE), if they were in normal distribution or median and interquartile range (IQR) if they were not in Gaussian distribution. Mann-Whitney *U* test was used to compare serum cytokines levels. The Wilcoxon's signed rank test was used to compare differences in the cytokine production of PBMCs. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. Cytokine and chemokine serum levels in patients taking glucocorticoids

To assess the effects of GC treatment on cytokine and chemokine serum levels, we first evaluated 52 SSc patients, of whom 19 (36.5%) were on systemic glucocorticoids treatment with prednisone. A mean dose of prednisone treatment was 3.8 mg/d (range 0–30 mg/d). Patients taking GC showed higher frequency of arthritis compared with patients not taking ($p < 0.0001$). There were not statistically significant differences in other clinical manifestations. (Table 2)

In most of SSc patients, serum levels of IL-2, IL-4, IL-10, TNF, and IL-6 were below the lowest detection limit. For IFN-γ and IL-17A, there was no difference in serum levels between patients taking or not taking corticosteroids. Also, we did not observe any significant differences in chemokines serum levels in patients taking corticosteroids compared to patients not taking (Table 3). In order to rule out the influence of systemic treatment, we evaluated the patients who were not taking any immunosuppressive treatment separately. There were no differences on serum levels of cytokines and chemokines between these group of patients (data not shown). We also assessed serum cytokines levels in patients taking only GC compared with patients treated with immunosuppressive drugs, but there was no significant difference (data not shown).

Effects of methylprednisolone treatment on chemokine production by PBMC from SSc patients and healthy controls after stimulation with anti-CD3/CD28