Glucocorticosteroid dependent decrease in the activity of calcineurin in the peripheral blood mononuclear cells of patients with systemic lupus erythematosus

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Abstract

Objectives—To compare the activity of calcineurin in the peripheral blood mononuclear cells (PBMC) of 32 patients with systemic lupus erythematosus (SLE) and 35 healthy controls.

Methods—The activity of calcineurin was assayed in the supernatants of sonicated mononuclear cells. On the other hand, the activity of calcineurin was reduced in patients with SLE taking GCS, correlating negatively with the dose of GCS. The stimulation of PBMC by phorbol ester and calcium ionophore decreased the calcineurin activity both in patients with SLE and in healthy controls. GCS could also reduce calcineurin activity in the mononuclear cells of healthy subjects in vitro.

Results—There was no significant difference in the calcineurin activity of patients with SLE not taking glucocorticosteroids (GCS) compared with the healthy controls. On the other hand, the activity of calcineurin was reduced in patients with SLE taking GCS, correlating negatively with the dose of GCS. The stimulation of PBMC by phorbol ester and calcium ionophore decreased the calcineurin activity both in patients with SLE and in healthy controls. GCS could also reduce calcineurin activity in the mononuclear cells of healthy subjects in vitro.

Conclusions—in patients with SLE the decrease in the calcineurin activity of PBMC depended on the dose of GCS used for treatment, and it was not a disease specific alteration. The higher the dose of GCS, the greater the inhibition of calcineurin activity. The reduction of calcineurin activity is a new element in the immunosuppressive effects of GCS during the treatment of patients with SLE.

Patients and methods

PATIENTS, CLINICAL DATA, AND CONTROL GROUP

The SLE study group comprised 32 patients, 29 women and three men, with a median age of 36 years (range 18–68). The mean SLE disease activity index (SLEDAI) was 4.1 (range 2–20). The average dose of GCS (methylprednisolone or equivalent of prednisolone) taken by the patients was 9.8 mg/day (range 0–32). These patients were free from any cytostatic drugs. The average duration of the disease was 8.5 years (range 0.5–35). Three subgroups of these patients were studied: (a) seven patients (six women, one man) not taking any GCS, median age 39 years (range 18–55), mean SLEDAI 4.4; (c) six patients (five women, one man) taking 8 mg of GCS/day, median age 39 years (range 19–52), mean SLEDAI 11.5. Thirty five healthy white subjects (32 women, three men), median age 36 years (range 20–52), served as controls. Approval was given through the institutional review board, and informed consent was obtained from all participants. All patients with SLE fulfilled the diagnostic criteria of the American College of Rheumatology with at least four of the revised criteria for the diagnosis of SLE. Patients with a SLEDAI score >3 were considered to have inactive disease.
Controls were taken as 100%. The number of patients is given in parentheses.

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Healthy controls

Patients with SLE

Figure 1 Negative correlation between the basal activities of calcineurin in the peripheral blood mononuclear cells (PBMC) of patients with systemic lupus erythematosus (SLE) and the doses of glucocorticosteroids (GCS) used for treatment. The coefficient of correlation between the basal calcineurin activities in the PBMC and the daily doses of GCS (0–32 mg/day) taken by the 32 patients was determined in five healthy control subjects. In these experiments the statistical significance was calculated as the difference between the basal activities in the presence and absence of Mn²⁺/Ca²⁺/calmodulin.

CULTURING OF PBMC IN THE PRESENCE OF PREDNISOLONE SODIUM SUCCINATE

PBMC of five healthy control subjects were cultured for 72 hours in the absence and presence of 10⁻⁸ M prednisolone sodium succinate (PMSF), 5 mM benzamidine, and 0.3% Triton X-100. The homogenate was centrifuged for 30 minutes at 10 000 g, and supernatants were collected and stored in aliquots at −70°C before the assays.

Calcineurin activity was measured by the release of ³²P, from ³²P labelled protein phosphatase inhibitor 1 (PPI) as described by Yang et al. with slight modifications. The assay mixture (30 µl) containing 50 mM Tris-HCl (pH 7.0), 0.3 mM dihydrothreitol, 0.2 mM CaCl₂, 1 mM MnCl₂, 0.04 mg/ml calmodulin or 2 mM EGTA, an appropriate amount of extract (0.5–1.5 mg/ml protein), and ³²P labelled protein phosphatase inhibitor 1 (12–25 000 cpm/reaction mixture) was incubated at 30°C for 10 minutes. All mixtures contained 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM benzamidine as protease inhibitors, and 20 nM okadaic acid as a protein phosphatase 2A inhibitor. The activity of protein phosphatase 1 was not assayed under the conditions given above, because 200 nM of okadaic acid did not inhibit the enzyme at all. The reaction was terminated by the addition of 100 µl of 10% trichloroacetic acid. ³²P of the supernatant fraction was determined after centrifugation at 10 000 g. The activity of calcineurin was calculated as the difference between the basal activities in the presence and absence of Mn²⁺/Ca²⁺/calmodulin.

RESULTS

The results show that the basal activity of calcineurin in the PBMC of patients with SLE and in healthy controls was compared by Student’s unpaired t test. During measurements of the in vitro effects of GCS on calcineurin activity, each value was calculated as the mean of the data from five healthy control subjects. In these experiments the statistical significance was calculated by Student’s paired t test. The correlation coefficient between the calcineurin activities and the doses of GCS was determined in the patients with SLE. In these studies the calcineurin activities of the individual patients were expressed as a percentage of the activities measured in the healthy controls, which were considered to be 100%.

NEGATIVE CORRELATION BETWEEN THE BASAL ACTIVITIES OF CALCINEURIN IN PBMC OF PATIENTS WITH SLE AND THE DOSES OF GCS USED FOR THE TREATMENT

The correlation between the basal calcineurin activity in the PBMC and the daily doses of GCS (0–32 mg/day) taken by the 32 patients.
with SLE was calculated. It was found to be highly significant: $r_s = -0.52$ ($p < 0.001$). The inhibition of calcineurin activity in the PBMC of patients with SLE may be a new factor in the immunosuppression caused by GCS. There was no significant difference in the calcineurin activities of seven GCS-free patients with SLE and those of 35 healthy controls (fig 1).

**Calcineurin Activities in the PBMC of Patients with SLE with or without GCS Treatment and in Healthy Controls**

In four groups of subjects the calcineurin activities of PBMC were assayed and compared. The cells were either non-stimulated or stimulated by PMA and Ca$^{2+}$ ionophore. In the non-stimulated cells of six patients with SLE taking 32 mg of GCS/day, there was a significant decrease in the calcineurin activity compared with the value of six healthy controls (9.7 mU/mg vs 15.1 mU/mg, $p < 0.05$). In the seven GCS-free patients or in the six patients taking 8 mg of GCS/day, the changes were not significant compared with the values of the healthy controls (14.6 mU/mg and 12.8 mU/mg vs 15.1 mU/mg). In the cells stimulated by PMA and Ca$^{2+}$ ionophore for four hours there was a marked decrease in the calcineurin activities of all groups compared with their

![Figure 2](image-url) Calcineurin activities in the peripheral blood mononuclear cells (PBMC) of patients with systemic lupus erythematosus (SLE) with or without glucocorticoid (GCS) treatment and in healthy controls. Calcineurin activities were assayed in the supernatants of non-stimulated and stimulated PBMC as described in "Patients and methods." The statistical significance of the differences was calculated by Student's unpaired t test. Asterisk denotes significant difference compared with the controls.

![Figure 3](image-url) In vitro effect of prednisolone sodium succinate (PRED) on the activity of calcineurin in peripheral blood mononuclear cells (PBMC) of healthy subjects. PBMC of five healthy controls were cultured for 72 hours in the absence and presence of $10^{-4}$ M PRED. Calcineurin activities were determined and the statistical analysis was carried out as described in "Patients and methods." Asterisk denotes significant difference compared with the respective control. For the calculation of statistical significance Student's paired t test was used.
non-stimulated counterparts: 5.2, 4.9, 4.5, and 3.8 mU/mg vs 15.1, 14.6, 12.8, and 9.7 mU/mg (p<0.001). The differences between the calcineurin activities of the stimulated cells in the four groups were not significant, but the changes showed a pattern similar to those found in the non-stimulated cells—the highest activity was measured in the healthy controls, the lowest in the patients receiving 32 mg of GCS/day (fig 2). In addition, we found no significant differences in the calcineurin activities of men or women with SLE taking or not taking GCS, but each group contained only one man (data not shown).

Discussion

The goal of these experiments was to measure the activity of calcineurin in the PBMC of patients with SLE. The major results are as follows. Firstly, there is no difference in the calcineurin activities of PBMC in the GCS-free cultures over the next two days: 26.7 and 21.6 mU/mg at 48 and 72 hours, respectively (p<0.001 and p<0.01). A similar tendency was seen in the cells treated with PRED. The activity of calcineurin increased significantly from the value at 24 hours (6.4 mU/mg) to 14.2 mU/mg and 12.9 mU/mg at 48 and 72 hours, respectively (p<0.001 and p<0.01). An assay of lactate dehydrogenase activities in the supernatants of cultured cells showed that neither the culturing nor the presence of PRED caused any cellular damage (data not shown). Thus the decreased calcineurin activity may be related to the effect of PRED.

There was no significant difference in the proportions of the various subsets of T cells in the patients with SLE and the controls. PRED was chosen for the in vitro experiments because the derivatives of prednisolone were mostly used also for the treatment of the patients with SLE. PRED was judged not toxic in these studies, according to the lactate dehydrogenase measurements. It is known that all GCS have both genomic and non-genomic membrane effects.13,14 According to these reports, the 32 mg/day dose of GCS in our patients and 10−4 M PRED applied in our in vitro experiments might have had a significant non-genomic influence, playing a part in the reduction of calcineurin activity. We found no decrease in the amount of calcineurin detected by immunoblotting (data are not shown) either in the cells of patients or in the cells of in vitro experiments treated with high doses of GCS, suggesting that the GCS induced inhibition of calcineurin activity was independent of the synthesis of the enzyme. However, in these cells an increased rate of apoptosis was detected at 24 and 48 hours of culturing compared with the GCS-free cells (data are not shown). This observation is in accordance with the earlier observation that the rate of apoptosis is increased in the T cells of patients with SLE treated with GCS.15 Therefore, we suppose that cabin 1, a newly discovered endogenous inhibitor of calcineurin, might possibly be one of the key molecules playing a part in this phenomenon, coupling the processes of steroid receptor regulation, apoptosis, and reduction of calcineurin activity in the PBMC.16 Furthermore, cabin 1 may also play a part in the reduced calcineurin activity seen in the cells stimulated by phorbol ester and Ca2+ ionophore in our experiments.

Our results confirmed the observation of Rider et al,17 who found no difference in the amounts of calcineurin mRNA in patients with SLE and healthy controls. According to our data this similarity was also reflected in the calcineurin activities of the PBMC of patients with SLE and healthy subjects. The significantly decreased calcineurin activity of PBMC measured in patients with SLE treated with 32 mg GCS/day, however, is a new observation. In addition, a significant negative correlation was found between the doses of GCS used in the treatment and the calcineurin activity (fig 1). This effect of GCS may also be an element of the immunosuppression related to the inhibition of the production of NF-AT dependent cytokines (for example, IL2, IL4, IL6, and IL13) in patients with SLE, and this pathway may coexist with other, well known influences of GCS.18 Calcineurin is a Ca2+ dependent enzyme, and, therefore, the inhibition of Ca2+ influx by GCS may have an important role in the GCS dependent decrease of calcineurin activity. This fact may partly explain the earlier finding that GCS inhibit the calcineurin dependent activation of IL23 and IL4 genes. However, the inhibition of calcineurin by GCS is not a phenomenon unique to GCS; it can be induced also in a dose dependent manner in the PBMC of healthy subjects in vitro.19
The mononuclear cells of healthy controls cultured in vitro showed rather different patterns of calcineurin activity in the untreated and PRED treated series (fig 3). The gradually increasing calcineurin activities of control cultures could be attributed to the production of several cytokines (for example, TNF or IL1) derived from the mononuclear cells attached to the surface of plastic dishes used for the culturing. By increasing the intracellular levels of Ca2+, TNF and IL1 may represent a new element in the immunomodulatory activity increased by the plastic surface, the calcineurin activity increased by the cytokine production of cultured cells during the second day of culturing (48 hours). As PRED can also inhibit the cytokine production of cultured cells activated by the plastic surface, the calcineurin activity increased by the effect of cytokines could never reach the values seen in the GCS-free counterparts of these cells.

Our data show that the decreased calcineurin activity induced by high doses of GCS may represent a new element in the immunosuppressive treatment of patients with SLE.

The authors thank Mrs Julia Hunyadi for expert technical assistance.
