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Circadian and concentration profile of cathepsin S in sera from healthy subjects and asthmatic patients

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Abstract Cathepsin S (CS) has been proposed to be associated with asthma pathogenesis but its exact role is not established. In order to understand this proposed association our objective was to follow the 24-h concentration pattern of CS in sera from apparently healthy subjects and from steroid-independent and steroid-dependent asthmatics before and after one weeks' treatment with methylprednisolone (MP) and cyclosporin A (CsA), respectively. Blood samples were collected every 4 h over a 24-h period. Statistical evaluation of data for time effect was performed by one way ANOVA and least-squares fit of 24-h cosine. Little or no significant change of CS concentrations with time over a 24-h period was observed in healthy and asthmatic sera. CS concentrations were significantly lower in steroid-independent asthmatics compared to controls while there was no difference between healthy subjects and steroid-dependent asthmatics. After one week of therapy MP decreased CS concentrations while CsA had no effect. Our data suggest the involvement of CS in asthma pathogenesis and the potential use of CS levels as an additional biological parameter for monitoring the extent of disease and response to therapy.

Key words asthma • cathepsin S • cyclosporin A • methylprednisolone • serum • 24-h variations

Introduction

CS is a lysosomal cysteine proteinase with significant elastolytic activity and extreme stability at neutral pH, thus accounting for its role outside lysosomes and participation in extracellular matrix remodelling [1]. CS is expressed specifically in lymphatic tissues and antigen presenting cells, indicating to have more specific physiological roles [2]. Its activity appears to be essential in the major histocompatibility complex (MHC) class II antigen presentation pathway and its expression is induced by cytokines such as interferon γ and interleukin 1β, which is an additional indication of its possible role in the immune response [2]. CS was found to be overexpressed at sites of arterial elastin damage [3]. Alterations in its expression and regulation could be associated with tissue remodelling or those pathological states exhibiting excessive immune response to exogenous antigens such as asthma. Our objective was to determine and compare CS concentrations in sera from healthy subjects and asthmatic patients, to establish its normal serum base-line circadian characteristics and any deviations with the disease and with MP and CsA therapy.

Materials and methods

Subjects and samples. Written informed consent was obtained from 21 volunteers participating in this study. The procedures were approved and performed in accordance with the guidelines of the regional medical ethics committee. Meals were served at 08:00, 12:30 and 18:00 h. Patients and healthy subjects were not restricted in their water intake, except for the half hour before sampling, but were asked to abstain from other liquids and food between the meals. Lights were switched off from 22:00 to 07:00 h. Each subject was required to maintain this sleeping and waking schedule for 2 days before study.

Group A consisted of 8 apparently healthy atopics (median age, 31 years; range, 22-63; 5 females, 3 men), without history of asthma and without any medication. The atopic status was defined with positive skin prick tests and/or with elevated total serum IgE concentration. All asthmatics had a typical history of asthma and documented reversibility of forced respiratory volume in one second (FEV_1) greater than 15 % after inhalation of bronchodilator. They had no acute or chronic infection, normal liver and renal function tests and they were lifelong non-smokers. *Group B* consisted of 8 steroid-independent atopic asthmatics (median age, 50 years; range 40-71; 4 women, 4 men) without regular use of the systemic corticosteroids. They had not received treatment with oral steroids for at least one month before the study or inhaled steroids for at least 3 days before the study. *Group C* consisted of the same 8 steroid-independent asthmatics after treatment with 40 mg oral MP daily for one week. *Group D* consisted of 5 steroid-dependent atopic asthmatics (median age, 61 years; range, 39-68; all women) with a continuous requirement for oral corticosteroid therapy over an average period of 13 ± 4 years (mean ± SE). Dose of oral corticosteroid (average daily dose of 18.6 mg; range, 16-32 mg) was not changed for at least 4 weeks before the study and remained the same through the study. Patients were also treated with inhaled β_2-agonists (salbutamol or fenoterol) prescribed as needed and oral sustained tablets of theophylline. Serum theophylline

concentrations were within therapeutic range (10-20 mg/l). Mean (SE) forced expiratory volume in one second (FEV_1) was 43 % (17 %) of the predicted value. *Group E* consisted of the same 5 steroid-dependent asthmatics after receiving CsA (Sandimmun^R, Sandoz Pharma Ltd., Basel) at a dose of 2 mg/kg/day for one week.

Blood was sampled on the day before and on the last day of the treatment at 4-h intervals during 24 h period beginning at 08:00 h. Blood samples of healthy subjects were collected on one day only. Blood was taken by venipuncture in the upright position according to National Committee for Clinical Laboratory Standards approved standard H3-A3. Blood was clotted at room temperature and centrifuged subsequently at 3000 rpm. Serum was separated, aliquoted and stored frozen at -20 °C until analysis.

Measurement of cathepsin S. Measurements were done with ELISA specific for CS (KRKA, d.d., Novo mesto, Slovenia), optimised (J. Kos *et al.*, unpublished results) and performed as suggested by the manufacturer. MAbs for CS recognised precursor and mature CS. Cross-reactivity between cysteine proteinases has been excluded. All determinations were performed in duplicate. A microplate reader Rainbow (SLT, Austria) was used to measure A_{450}. The measured values of diluted samples in the ratio 1:2 were subsequently compared with the calibration curve and expressed in ng/ml of serum. The detection limit was 0.8 ng/ml.

Statistical methods. All data were analysed with values in original units and transformed to percentage of individual 24-h means in order to minimise interindividual differences. Since one-way ANOVA, which validated any apparent differences by comparing different timepoints, can fail to obtain the actual high point of the rhythm, data were analysed also individually and as a group for circadian rhythm by single and population mean cosinor analysis[4]. Cosinor analysis involves the fit of a 24-h cosine curve by the method of least squares, and provides both the probability of rejection of the null amplitude hypothesis for a chosen period (24 h in this case) and the rhythm characteristics: the mesor (24-h adjusted means), the amplitude (half the difference between the maximum and the minimum fitted cosine function), and the acrophase (time of maximum in fitted cosine function, with midnight as the phase reference). Group comparisons were made by the Wilcoxon matched-pairs signed-ranks test and by Mann-Whitney U-Wilcoxon rank sum W test. The correlations were analysed by Spearman rank test. Two-sided P values < 0.05 were considered significant.

Results

Overall time point means ± 2SE of CS in healthy and asthmatic sera over 24 h are shown in Fig. 1.

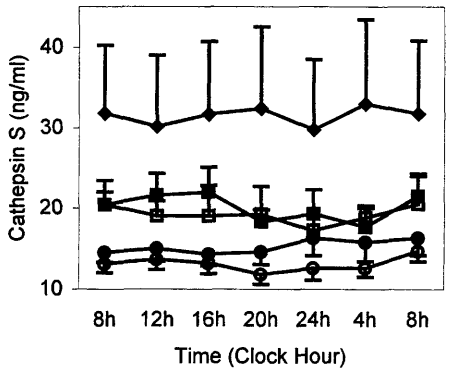

Fig. 1 Circadian variations of serum CS (24-h mean ± 2 SE) in healthy subjects (♦), steroid-independent asthmatics before (●) and after (○) MP treatment, and in steroid-dependent asthmatics before (■) and after (□) CsA treatment.

Table 1 shows the mean 24-h concentrations, the range between maximum and minimum concentrations over 24h of CS, ANOVA and population mean cosinor analysis. Inter-individual differences were validated with statistical significance ($P < 0.0001$) in all study groups.

Table 1 Circadian characteristics for serum CS measured every 4 h for 24 h in healthy subjects (A), steroid-independent asthmatics before (B) and after (C) treatment with MP, steroid-dependent asthmatics before (D) and after (E) treatment with CsA*.

				ANOVA		Least-squares fit of 24-h cosine			
Group	**Units**	**24-h mean ± 2 SE**	**Range**	**F**	**P**	**P**	**Mesor ± SE**	**Amplitude ± SE**	**Acrophase ± SE (h)**
A	ng/ml	30.9 ± 8.9	15.0 – 86.7	0.02	1.0	0.04	30.8 ± 8.9	0.8 ± 0.2	09:46 ± 01:35
	% of mean	24 ± 5	5 – 48	2.6	0.03	0.4	112 ± 13	29	08:12
B	ng/ml	15.2 ± 1.5	10.6 – 22.6	0.2	1.0	0.3	15.0 ± 1.5	1.2	02:53
	% of mean	54 ± 13	11 - 123	1.0	0.5	0.3	99 ± 1	6	03:03
C	ng/ml	12.9 ± 1.1	8.1 – 17.6	0.5	0.8	0.2	12.9 ± 1.1	0.8	10:39
	% of mean	41 ± 6	15 - 68	1.6	0.2	0.2	99 ± 0.2	6	10:07
D	ng/ml	19.7 ± 2.2	13.6 – 25.9	0.5	0.8	0.2	19.7 ± 2.1	1.7	12:26
	% of mean	45 ± 5	36 - 64	1.8	0.1	0.08	100 ± 1	9	12:46
E	ng/ml	19.1 ± 1.7	12.7 – 21.9	0.3	1.0	0.003	19.3 ± 1.7	0.8 ± 0.1	11:04 ± 01:46
	% of mean	38 ± 11	15 - 78	1.0	0.5	0.02	99 ± 1	5 ± 3	09:56 ± 01:49

*Statistical evaluation for circadian time effect and rhythm was determined by one-way ANOVA and population mean cosinor analysis. Range, the difference between the maximum and minimum concentrations over 24 h. % of mean, data transformed to percentage of individual 24-h means.

This led us to perform temporal analysis also on data transformed as percentage of the individual's 24-h mean (Table 1). Using single cosinor analysis CS revealed significant circadian variations on an individual level only in one subject of group D ($P = 0.008$; mesor ± SE, 25.7 ± 0.5 ng/ml; amplitude ± SE, 4.4 ± 0.7 ng/ml; acrophase ± SE, 13:08 ± 00:34h) and in one subject of group E ($P = 0.04$; mesor ± SE, 12.4 ± 0.5 ng/ml; amplitude ± SE, 2.6 ± 0.7 ng/ml; acrophase ± SE, 06:52 ± 01:02h).

No significant correlation was found between the 24-h concentration changes of serum CS and cathepsins B, H, and L, stefins A and B, and cystatin C ($P > 0.5$), determined

earlier in the same serum samples from healthy subjects by specific ELISAs [5,6].

Group comparisons of overall CS concentrations were not significant between the following groups: A:D ($P = 0.3$), A:E ($P = 0.4$) and D:E ($P = 0.2$). Compared to controls CS concentrations were significantly decreased in steroid-independent asthmatics before and after MP therapy (A:B and A:C, $P < 0.0001$). Significant differences were observed between pre- and post-therapy sera of steroid-independent asthmatics (B:C, $P = 0.0001$) where the effect of MP was demonstrated. CS concentration was significantly increased in steroid-dependent asthmatics with pre-existing more severe degree of disease, in contrast to steroid-independent asthmatics (B:D, $P = 0.0001$).

Discussion

In this study we followed concentration and circadian pattern of CS in the serum samples from healthy subjects and asthmatic patients (Fig. 1). When we compared the 24-h changes of normal serum CS with those of other cysteine proteinases, cathepsins B, H and L, determined earlier in the same normal sera, no significant correlation was found to indicate a possible similar regulation of expression as was suggested before for cathepsins H and L [5]. Temporal analysis of CS revealed significant circadian rhythms only in healthy subjects (A) and steroid-dependent asthmatics after CsA treatment (E). However both rhythms had small amplitudes, ranging from 2 to 29% of the 24-h mean, with both acrophases localised in the morning (Table 1). We have demonstrated previously that the serum levels of cathepsins B and H, and their low molecular weight inhibitors, cystatin C, stefins A and B, showed no significant circadian variations in apparently healthy subjects; only cathepsin L showed a small amplitude [5,6]. Recently we have observed little or no significant changes with time of cystatin C concentrations over a 24-h period in sera from asthmatic patients [7].

CS concentrations showed a tendency to be lower in asthmatic sera compared to controls. However, group comparisons demonstrated that this difference was significant only between healthy and steroid-independent asthmatics. On the other hand the concentrations of cystatin C, the most abundant extracellular inhibitor of cysteine proteinases, were significantly elevated in sera of asthmatic patients [7]. Since asthma is characterised by exaggerate immune responses the balance between CS and cystatin C may be important because of the proposed role of CS in antigen presentation [2]. Cystatin C has already been reported to regulate maturation of MHC class II molecules during antigen presentation in bone-marrow-derived dendritic cells by inhibiting CS [8].

After one weeks' treatment with low dose oral MP, serum CS concentration was decreased while no effect was observed after CsA therapy. To our knowledge this is the first report of the drug effect on serum CS concentrations in a clinical study.

We can conclude that CS temporal differences in healthy and asthmatic sera are too small which could influence the test results obtained in the course of a day, underlying its potential usefulness as an additional biological marker. Further, decreased CS concentrations together with increased cystatin C concentrations [7] in asthmatic sera suggest that they both may be involved in the pathogenesis of asthma.

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