

Circadian rhythms of cysteine proteinases and cystatins, potential tumour markers, in normal sera

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Introduction

Circadian day/night variations have been evidenced in all major groups of organisms and at all levels of organisation of the organism. Circadian intra-individual variations are known for a number of analyses in serum including tumour-associated markers.¹⁻⁴ It was suggested that the serum levels of cysteine proteinases and their inhibitors may be of clinical importance for prognosis and diagnosis in cancer.⁵ Since known circadian rhythms are important for choosing the best sampling time, interpretation of the results of a diagnostic test, patient monitoring, and timing of a therapy, our objective was to establish 24-h variations of cysteine proteinases, cathepsins B, H, L, and their low molecular weight inhibitors, stefin A, stefin B, and cystatin C, in sera from healthy subjects.

Materials and methods

This study, which included eight clinically symptom-free adults (median age, 31 years; range, 22-64 years; 5 females, 3 men), was performed as reported previously.^{6,7} Before entering the study the volunteers had given

informed written consent, and 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. The lights were switched off from 22:00 to 07:00 h.

Blood was taken by venipuncture in upright position according to National Committee for Clinical Laboratory Standards approved standard H3-A3. Seven samples were collected at 4-hour intervals beginning at 08:00 h. Blood was clotted at room temperature and centrifuged subsequently at 3000 rpm. Serum was separated, aliquoted and frozen at -20 °C until analysis.

Measurements of all proteins were done by specific ELISAs (Krka, d.d., Novo mesto, Slovenia) as described previously.^{8,9}

Mean values and SE were computed at fixed hours for each subject during the 24-hour monitoring. All data were analysed by one-way ANOVA and by cosinor analysis involving the fit of a 24-hour cosine curve by the method of least squares^{10,11} as reported previously.^{6,7} The correlation between the parameters examined was assessed by the nonparametric Spearman rank correlation test. Two-sided P values < 0.05 were considered significant.

Results and discussion

The 24-h patterns of cathepsins B, H, L, cystatin C, stefins A and B were investigated in sera of clinically healthy subjects. To minimise the factors such as posture, activity, food ingestion, stress, sleep or wakefulness, which could contribute to the variations, the subjects were required to maintain the same regime during the study and 2 days before the beginning. All tested proteins in normal sera were in the nM concentration range and among them cystatin C was the most abundant since only cystatin C is localised extracellularly (Table 1). Common feature of 24-hour patterns was that cystatins and cathepsins reached their minimal values in the resting period, except for cathepsin B and stefin B, which were close to the daily mean throughout the day. Comparing all patterns of cathepsins and cystatins a significant relationship between the variations was observed only for cathepsins H and L, indicating possible similar regulation of expression.

To eliminate between individual variability, each individual's data were transformed to deviation from that individual's 24-hour

mean. All data were subjected to ANOVA, which validated any apparent differences by comparing different time points. Since ANOVA 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, which involves the fit of a 24-hour cosine curve by the method of least squares. The method provides both the probability of rejection of the zero amplitude hypothesis for a chosen period (24 h in our case) and rhythm characteristics: the mesor (24-hour 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). ANOVA revealed no significant time effect except for transformed data of cathepsins H and L and both stefins (Table 1). Using single cosinor analysis no significant rhythm was revealed, except for cystatin C, stefin A and cathepsin H, where only one subject demonstrated a circadian variation ($P \leq 0.04$). On the group level using population mean cosinor analysis, the patterns of all investigated serum prote-

Table 1. Circadian characteristics for serum cathepsin B (CB), cathepsin H (CH), cathepsin L (CL), cystatin C (CC), stefin A (SA) and stefin B (SB), measured every 4 hours for 24 hours in healthy subjects. 24-h means with range between subjects are presented. Statistical evaluation for circadian time effect and rhythm was determined by ANOVA and cosinor analysis.

Serum protein	Units	24-h mean ± 2 SE	Range	ANOVA			Least-squares fit of 24-h cosine		
				F	P	P	Mesor ± SE	Amplitude ± SE	Acrophase ± SE (h)
CB	ng/ml	4.5 ± 0.5	3.5 - 7.5	0.02	1.0	0.7	4.5 ± 0.5	0.1	08:40
	% of mean	19 ± 2	11 - 26	0.4	0.9	0.8	100 ± 0.2	1	06:55
CH	ng/ml	15.0 ± 2.2	7.8 - 24.4	0.4	0.9	0.1	14.8 ± 2.2	1.6	11:51
	% of mean	112 ± 51	18 - 440	2.8	0.02	0.2	99 ± 1	15	11:13
CL	ng/ml	18.1 ± 2.7	7.5 - 32.3	0.5	0.8	0.02	18.0 ± 2.7	2.1 ± 0.5	11:38 ± 00:41
	% of mean	66 ± 17	7 - 113	6.6	<0.001	0.03	99 ± 0.3	15 ± 4	12:01 ± 00:47
CC	ng/ml	681.9 ± 39.4	438.2 - 1156.0	0.4	0.9	0.2	675.5 ± 76.2	40.2	07:19
	% of mean	60 ± 9	27 - 90	1.9	0.1	0.2	99 ± 0.3	5	07:23
SA	ng/ml	6.1 ± 0.4	4.2 - 8.2	1.0	0.4	0.2	6.1 ± 0.4	0.3	14:26
	% of mean	85 ± 7	32 - 77	3.1	0.01	0.2	100 ± 1	6	14:34
SB	ng/ml	3.0 ± 1.2	0.5 - 8.8	0.05	1.0	0.1	3.0 ± 1.2	0.1	06:52
	% of mean	49 ± 7	19 - 86	4.3	0.002	0.2	100 ± 0.3	5	03:46

ins showed no significant circadian rhythm with the exception of cathepsin L where the rhythm exhibited a small amplitude, ranging from 5-24 % of the 24-hour mean, and an acrophase localised at around 12 h (Table 1).

Conclusion

We conclude that the time of sampling in the course of day has a minor influence on measurements of cathepsin L, and none on cathepsins B and H, stefins A and B, and cystatin C in normal sera which underlines their usefulness as potential clinical markers. The possible changes in their circadian structure with different types of cancer will be of considerable interest.

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