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Stability of YKL-40 concentration in blood samples

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The stability of YKL-40, a mammalian member of the family of 18 glycosyl-hydrolases, in blood samples handled under different temperatures and different time intervals before centrifugation was studied in paired serum and plasma samples from 25 healthy premenopausal Danish women. Significant elevations of YKL-40 were found in 8 paired serum samples left on the clot for more than 3 h at room temperature compared to paired serum samples left on the clot for 3 h or less. Significant elevations of YKL-40 were found in 8 paired plasma (EDTA) samples left on the blood cells for more than 8 h at room temperature compared to paired plasma (EDTA) samples left on the blood cells for 8 h or less. No elevations were found in YKL-40 levels in serum samples left on the clot at 4°C for 24 h or in plasma (EDTA) samples left on the blood cells for 72 h before centrifugation. Significantly lower concentrations of YKL-40 were measured in plasma (EDTA) compared with paired serum samples with a serum/plasma ratio of 1.4 in samples left on the clot or on blood cells at 4°C for up to 24 h. Repetitive freezing and thawing had no significant effect on the measured YKL-40 concentrations. In conclusion, we have shown that YKL-40 is very dependent on the handling procedures. All the blood samples must be processed into plasma (EDTA) within 8 h at room temperature or into serum in less than 3 h at room temperature. If this is not possible, the blood samples must be stored at 4°C until processed.

Key words: ELISA; plasma; serum

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INTRODUCTION

YKL-40 is a member of the 18 glycosyl-hydrolase family [1, 2], a protein family which includes chitinases and chitinase-related proteins. YKL-40 is a lectin that binds chitin [3] and heparin [4], but it has no chitinase activity [2, 3]. The protein has been termed YKL-40 from its molecular weight (40 kDa) and the one-letter code for its three N-terminal amino acids [5] and is also named human cartilage glycoprotein-39 [2]. The sequence of the gene has been published, but promotor analysis and regulatory factors have not been described [6]. Although the physiological function of YKL-40 is unknown, the pattern of its expression in the normal and disease state suggests a role in remodelling or degradation of the extracellular matrix. YKL-40 is secreted in large amounts *in vitro* by the MG63 human osteosarcoma cell line [5] and as a protein expressed by murine mammary tumours initiated by neu/ras oncogenes [7].

Recently, it has been demonstrated that the serum YKL-40 level is elevated in patients with colorectal cancer and in patients with recurrent metastatic breast cancer compared to the YKL-40 level in healthy controls. In both studies a significant association between increased serum YKL-40 level and short survival was found. Serum YKL-40 may therefore be used as a prognostic biochemical marker of survival [8, 9]. We therefore planned to include YKL-40 measurement in an ovarian cancer study. Because the study involved blood sampling at different hospitals and transport of the blood samples to a central laboratory, we undertook this study to assess the stability of YKL-40 in the 1–3 day time period required for postal delivery.

MATERIALS AND METHODS

Subjects

The study population consisted of 25 healthy premenopausal women from Denmark. The blood samples were collected in three different sets. One set consisted of serum samples from 8 women (set A), another of serum and paired plasma samples (EDTA and heparin) from 9 women (set B), and a third set of serum and plasma (EDTA) samples from 8 women (set C). The collection tubes, the storage temperature before centrifugation and the length of storage before centrifugation were changed between the different groups of samples. All samples were then centrifuged at $2000 \times g$ for 10 min, and the plasma or serum samples were stored at -20°C until YKL-40 analysis was performed. In addition, to investigate the effect of repetitive thawing and freezing the serum from 9 women (set B) was divided into 8 aliquots and frozen within 1 h from venipuncture. After 24 h, seven aliquots from each woman were thawed at room temperature and refrozen after 1 h. This process was repeated daily, leaving one aliquot frozen each day, until the last aliquot was thawed and refrozen after 7 days before YKL-40 analysis.

Laboratory analysis

Serum and plasma YKL-40 concentrations were determined in duplicates using an ELISA system developed by Harvey *et al.* [10] in accordance with the manufacturer's instruction (Metra Biosystems, Mountain View, CA, USA). The sensitivity of the assay was 20 μg L^{-1} and the intra- and interassay variations were 3.6% and 5.3%, respectively ($n=67$).

Statistical analysis

All distributions were examined for normality before the application of parametric analyses. A paired *t*-test was used for data examination.

RESULTS

At room temperature a minor decrease in YKL-40 mean level in 8 plasma (EDTA) samples (set C) left on the blood cells for 3 h was seen compared to samples left on the blood cells for 1 h ($p=0.05$). In contrast, a non-significant increase was found in plasma (EDTA) samples left on the blood cells for 8 h at room temperature before separation compared to samples left for 1 h ($p=0.77$). For plasma (EDTA) samples left on blood cells at room temperature for 24 and 72 h before plasma pipetted off the blood cells, the increases in the YKL-40 levels were significant compared to samples left on the

TABLE I. YKL-40 levels (μg L^{-1}) in plasma (EDTA) left on the blood cells at respectively 4°C and at room temperature (24°C) for 1 h, 3, 8, 24 and 72 h before samples are centrifuged and plasma pipetted off the blood cells (set C).

Temperature	4°C					24°C				
Hours clotting	1	3	8	24	72	1	3	8	24	72
Mean	25.4	26.0	25.8	26.9	26.6	25.5	24.1	25.8	32.0	36.6
Range	20–35	20–37	20–36	20–38	20–37	20–36	20–34	20–38	21–45	23–53
SD	5.4	5.9	5.5	6.4	6.4	5.7	4.7	6.0	8.7	10.3
P values*		0.57	0.68	0.16	0.18		0.05	0.77	0.003	0.002

Number of samples repetitively analysed = 8. SD = standard deviation. * The p values are the significance levels between plasma at 1 h and the actual samples at 3, 8, 24 or 72 h.

blood cells for only 1 h (24 h: $p = 0.003$; and 72 h: $p = 0.002$) (Table I). There were no significant changes in YKL-40 levels in plasma (EDTA) samples left on the blood cells at 4°C for respectively 3, 8, 24 and 72 h compared to samples left on the blood cells for 1 h (3 h: $p = 0.57$; 8 h: $p = 0.68$; 24 h: $p = 0.16$; and 72 h: $p = 0.18$) (Table I).

A small increase in serum YKL-40 concentrations was observed in 8 samples (set A) left on the clot at room temperature for 3 h ($p = 0.054$), but the elevation in serum YKL-40 was highly significant after 72 h ($p = 0.004$) compared with paired serum samples separated by centrifugation followed by pipetting off the clot 1 h after venipuncture (Table II). No difference was found in serum YKL-40 levels between samples left on the clot at 4°C for 1 h and 3 h ($p = 0.13$) (Table II). Significant increases were found in serum YKL-40 levels when samples left on the clot at 4°C for 1 h were compared to paired samples left on the clot at 4°C for 72 h ($p = 0.007$) (Table II). In the third set of samples from 8 healthy women (set C) the serum YKL-40 levels were stable in samples left on the clot at 4°C for 8 h and 24 h compared to samples centrifuged and pipetted off the clot after 1 h (8 h: $p = 0.32$; and 24 h: $p = 0.15$). In the paired samples left on the clot at room temperature, significant increases in serum YKL-40 levels were found after 8 h ($p = 0.001$) and 24 h ($p = 0.001$) compared to samples left on the clot for only 1 h before centrifugation and separation off the serum from the clot by pipetting.

YKL-40 levels were significantly higher in serum compared to paired plasma samples (serum vs. EDTA plasma: 55.8 vs. 38.8 μg L^{-1}, $p = 0.003$; and serum vs. heparin plasma: 55.8 vs. 43.8 μg L^{-1}, $p = 0.004$) centrifuged after 1 h at room temperature. The lowest YKL-40 levels were found in EDTA plasma compared to the corresponding serum or heparin levels. The serum/plasma ratio of YKL-40 was 1.4 after 1 h, 8 and 24 h at 4°C, but increased in serum and plasma samples that had been left on the clot or left on the blood cells at room temperature for 8 and 24 h (Table III).

Repetitive freezing and thawing of the serum up to eight times did not significantly affect the serum YKL-40 levels in paired samples (set B) (Table IV).

TABLE II. YKL-40 levels (μg L^{-1}) in serum samples left on the clot at 4°C and at room temperature (24°C) in 1 h, 3 and 72 h before separation off the serum from the clot (set A).

Temperature	4°C			24°C		
Hours clotting	1	3	72	1	3	72
Mean	49.3	44.9	54.8	50.3	53.1	97.3
Range	20–111	20–98	20–118	20–111	20–113	20–158
SD	30.26	26.28	30.99	30.17	30.38	49.06
P values*		0.13	0.007		0.054	0.004

Number of samples repetitively analysed = 8. SD = standard deviation. * The p values are the significance levels between serum at 1 h and the actual samples at 3 h or 72 h.

TABLE III. The ratios of YKL-40 levels (μg L^{-1}) in serum compared to plasma (EDTA) separated off the clot or off the blood cells after respectively 1 h, 8 and 24 h at 4°C and at room temperature (24°C).

Temperature	4°C			24°C		
Hours clotting	1	8	24	1	8	24
Mean ratio	1.41	1.42	1.42	1.61	1.95	2.03
Range ratio	0.83–1.79	1.10–1.83	1.25–1.85	1.25–1.90	1.40–2.74	1.33–2.66
SD ratio	0.29	0.27	0.21	0.24	0.42	0.45
P values*		0.93	0.92		0.07	0.037

Number of samples repetitively analysed = 9. SD = standard deviation. * The *p* values are the significance levels between the serum/plasma ratio 8 and 24 h and the ratio at 1 h.

DISCUSSION

We found levels of YKL-40 to be sensitive to different handling conditions. In serum left on the clot at 4°C the YKL-40 levels were stable for up to 24 h, but after 72 h the serum level was significantly increased. The YKL-40 levels were stable in plasma (EDTA) left on the blood cells for 72 h when stored at 4°C before centrifugation followed by plasma pipetted off the blood cells. The most important finding of the present study was seen in blood samples left at room temperature before separation into serum or plasma by centrifugation. We found significant increases in YKL-40 levels in serum (55% increase) and in plasma (EDTA) (25% increase) left for 24 h before separation compared to the levels in serum and plasma samples left on the clot or left on the blood cells for 1 h at room temperature before separation. These findings may seriously influence the specificity of YKL-40 in clinical studies, where the samples are transported from the sampling place to a central laboratory. Blood samples drawn for the YKL-40 analysis should therefore be processed into serum within 3 h or for EDTA-plasma samples in less than 8 h. If the blood samples have to be transported for a longer time period before centrifugation and separation of serum or plasma, the samples should be stored and shipped at 4°C.

The most likely explanation for the time-dependent increase in YKL-40 levels at room temperature is that a degranulation of neutrophils occurs in the blood sample. YKL-40 is present in the specific granules of human neutrophils isolated from the blood of healthy subjects [11] and is released from these granules by exocytosis. It is well known that neutrophils degranulate in blood samples during prolonged storage and release the proteins in the granules [12, 13]. The YKL-40 levels were higher in serum compared to plasma (EDTA and heparin) and this is in accordance with other studies that have reported significantly higher levels of lactoferrin and neutrophil gelatinase-associated lipocalin NGAL (proteins present in the specific granules of neutrophils) in serum compared with plasma [13]. The differences between serum and plasma YKL-40 levels have to be considered when reference intervals are established in future studies.

Serum samples were stable on repetitive freezing and thawing up to eight times. This is re-assuring, as in many studies the samples are frozen for later analysis. Commonly, frozen serum samples also may have been thawed and frozen several times for other analyses when

TABLE IV. The influence of freezing and thawing on the YKL-40 serum levels (μg L^{-1}) (set B).

No. of freeze cycles	1	2	3	4	5	6	7	8
Mean YKL–40	55.8	52.1	52.8	53.1	52.6	55.0	51.4	53.6
Range	30–82	30–91	33–78	30–85	30–80	33–91	28–84	32–84
SD	15.73	17.67	12.32	16.14	14.62	18.33	15.73	15.45
P values*		0.21	0.15	0.20	0.25	0.81	0.13	0.42

Number of samples repetitively analysed = 9. SD = standard deviation. * The *p* values are the significance levels between one freezing and the actual freezing number (e.g. No. 1 vs. No. 5: $p = 0.25$).

new tumour marker assays appear. Earlier and future YKL-40 studies performed on correctly processed, frozen and thawed samples will still be trustworthy.

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