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ORIGINAL ARTICLE

Instability of Plasma and Serum Progastrin-Releasing Peptide During Repeated Freezing and Thawing

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Abstract
Objectives: Progastrin-releasing peptide (proGRP) is a promising biomarker for small cell lung cancer. However, not much is known about how sample processing and storage conditions affect the stability of proGRP. Here, we examined the effects of repeated freeze–thaw cycles on the stability of proGRP in plasma and serum.
Methods: Concentrations of proGRP were measured in plasma and serum samples exposed to two, three, or four freeze–thaw cycles and these were compared with values of corresponding samples exposed to one cycle (baseline). We also performed the area under the receiver-operating-characteristic curve (AUC) analysis to determine whether the differences of proGRP concentrations between each paired plasma and serum sample (ΔproGRP) can be used for identifying the samples that have been exposed to multiple freeze–thaw cycles.
Results: Concentrations of proGRP gradually decreased in both plasma and serum samples with increasing numbers of freeze–thaw cycles. Reduction rates of proGRP concentrations were greater in serum than in plasma samples and serum proGRP levels declined with statistical significance ($p < 0.001$) up to 10.1% after four freeze–thaw cycles. The ΔproGRP measurement showed fair accuracy (AUC $= 0.741$) for identifying samples that had been through four freeze–thaw cycles. The sensitivity was 82.8% and specificity was 62.1% at an optimal cut-off point of > 4.9.
Conclusion: Our study shows that the stability of circulating proGRP is affected in both plasma and serum samples by repeated freezing and thawing. We also show that ΔproGRP could be used for identifying paired plasma and serum samples subjected to multiple freeze–thaw cycles.

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1. Introduction

Recently, circulating progastrin-releasing peptide (proGRP) has been reported as a putative biomarker of small cell lung carcinoma (SCLC) [1−4]. ProGRP is a precursor of gastrin-releasing peptide (GRP), a gastrointestinal hormone of the bombesin family [5, 6]. GRP cannot be used as a biomarker due to its instability (the half-life of GRP is < 3 min) [7]. However, proGRP is a stable protein with half-life of 19−28 days [8]. An assay for serum proGRP showed high sensitivity (47−86%) and specificity (95−100%) in diagnosing SCLC [1−4]. Testing for plasma proGRP also exhibited high sensitivity (> 80%) and specificity (> 90%) [9].

Circulating proteins can be changed by pre-analytical treatments such as sample collection, processing, and cryopreservation conditions [10−13]. To achieve accurate disease diagnosis using circulating proGRP, it is important to identify sample collection, processing, and storage variables influencing the stability of proGRP and to establish reliable protocols. Effects on the stability of circulating proGRP are especially important in the collection and management of biobank samples that can be used for future research. Biobank samples may undergo multiple freeze−thaw cycles because of a limited number of aliquots [14], thereby inducing the instability of circulating proteins [15,16]. Previous studies showed that proGRP concentrations were changed in plasma and serum samples with increasing storage time at 2−6°C or room temperature [17−19]. Serum proGRP concentration was observed to decrease with the number of freeze−thaw cycles [19]. However, that study was limited because it involved samples from only two participants.

In this study, we used dozens of plasma and serum samples to investigate whether detection of circulating proGRP is changed by repeated freezing and thawing of samples. Furthermore, we analyzed the data using area under the receiver-operating-characteristic curve (AUC), to determine whether ΔproGRP can be used to identify repeated freezing and thawing of paired plasma and serum samples.

2. Materials and methods

2.1. Sample preparation

Whole-blood samples were collected from 30 healthy volunteers consisting of 15 men (aged between 27 years and 41 years) and 15 women (aged between 25 years and 52 years). All volunteers provided written informed consent. Plasma separator tubes (K_2 EDTA tubes; Becton Dickinson, Franklin Lakes, NJ, USA) and vacutainer serum separator tubes (SST tubes; Becton Dickinson) were used to collect the whole blood. Within 20 minutes after whole-blood collection, all tubes were centrifuged for 15 minutes at 2,000*g* at 4°C, to obtain plasma and serum samples. Each of the plasma and serum samples was aliquoted into four 1.5 mL tubes. Each aliquot of plasma or serum was repeatedly frozen at −70°C and thawed at 37°C one, two, three, or four times. The thawing time at 37°C and exposure time at room temperature were recorded.

2.2. Measurement of proGRP concentrations

Levels of proGRP (pg/mL) in plasma and serum samples were measured, in duplicate, with Architect ProGRP immunoassays (Abbott Japan, Tokyo, Japan) in accordance with the manufacturer's protocol.

2.3. Statistical analysis

Concentrations of proGRP are shown as mean ± standard deviation. Concentration changes of proGRP under repeated freezing and thawing conditions (2, 3, and 4 freeze−thaw cycles) are expressed as mean percentage changes with a "+" for increase and a "−" for decrease, compared with one freeze−thaw cycle (baseline). The statistical significance of proGRP changes was estimated via paired two-tailed *t* tests using SPSS statistical software, Version 13 (SPSS Inc., Chicago, IL, USA).

Receiver-operating-characteristic (ROC) curve analysis was performed using MedCalc software for Windows (MedCalc Software, Ostend, Belgium). The differences of proGRP concentrations between the paired plasma and serum samples (plasma proGRP concentration − serum proGRP concentration; ΔproGRP) that were exposed to one freeze−thaw cycle and those exposed to four freeze−thaw cycles were used for this analysis. The results of AUC analysis were considered excellent for AUC values > 0.9, good for AUC values between 0.8 and 0.9, fair for AUC values between 0.7 and 0.8, and poor for AUC values < 0.7. In all statistical analysis, p values < 0.05 were regarded as statistically significant.

3. Results

3.1. Effect of repeated freezing and thawing of plasma and serum samples on the stability of proGRP

Concentrations of proGRP were measured in plasma and serum samples exposed to two, three, and four freeze−thaw cycles, and the values were compared with concentrations of proGRP in corresponding samples that had undergone one freeze−thaw cycle (baseline). Concentrations of proGRP in both plasma and serum samples showed a tendency to decease with repeated freezing and thawing (Table 1). The reductions of proGRP concentrations in serum samples were greater than those in plasma samples. Serum proGRP concentrations decreased with statistical significance (p < 0.001) up to 10.1% after four freeze−thaw cycles (Table

1). The differences of proGRP concentrations between each pair of plasma and serum samples (ΔproGRP), which were exposed to the same freeze−thaw cycle, increased with increases in the number of freeze−thaw cycles (Figure 1). As shown in Table 2, the thawing time at 37°C and exposure time at room temperature were similar between plasma and serum samples, meaning that differences of proGRP concentrations between paired plasma and serum samples were not induced by the experimental technique.

3.2. Accuracy analysis of proGRP levels in the assessment of past freezing and thawing of plasma and serum samples

We performed ROC curve analysis to identify whether proGRP levels can be used for assessing the repeated freezing and thawing history of plasma and serum samples. For this analysis, we used ΔproGRP in samples exposed to one and four freeze−thaw cycles; ΔproGRP showed fair accuracy (AUC = 0.741) for discriminating paired plasma and serum samples exposed to four freeze−thaw cycles (Figure 2) compared with those subjected to only one cycle. Additionally, the sensitivity was 82.8% and specificity was 62.1% at an optimal cutoff point of > 4.9.

3.3. Levels of proGRP in healthy individuals

Concentrations of proGRP were measured using plasma and serum samples from 30 healthy individuals. As shown in Table 1, plasma proGRP concentrations ranged from 27.6 pg/mL to 91.6 pg/mL (mean, 41.3 pg/mL; standard deviation, 14.9 pg/mL) and serum proGRP concentrations ranged from 24.3 pg/mL to 83.4 pg/mL (mean, 36.5 pg/mL; standard deviation, 13.6 pg/mL), when they were measured after one freeze−thaw cycle. Although these values are not equivalent to those from fresh samples, our data contribute to the knowledge of the reference ranges for circulating proGRP in healthy individuals.

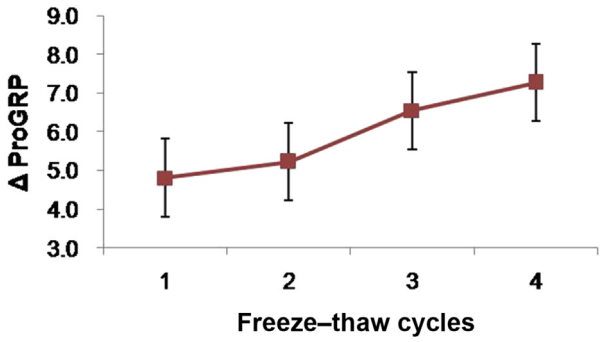

Figure 1. Differences of proGRP concentrations between paired plasma and serum samples (ΔproGRP). proGRP = progastrin-releasing peptide; ΔproGRP = plasma progastrin-releasing peptide concentration−serum progastrin-releasing peptide concentration.

4. Discussion

ProGRP is a putative biomarker for SCLC, a subtype of lung cancer [1−4]. In this study, we assessed the effects of multiple freeze−thaw cycles on the stability of proGRP in plasma and serum. Concentrations of proGRP decreased with statistical significance in both plasma and serum samples that were repeatedly frozen and thawed two, three, and four times, as compared to baseline (one freeze−thaw cycle) values. In a previous study, proGRP concentrations were significantly reduced by repeated freezing and thawing of serum samples but this was not found in plasma samples [19]. The difference between our results may be due to experimental variables such as sample size and thawing conditions. Serum proGRP levels declined up to 10.1% after four freeze−thaw cycles. During repeated freeze−thaw cycles, serum samples were incubated at 37°C and room temperature for 11.6 minutes. It has been reported that proGRP concentrations in serum samples stored for 2 hours at room temperature were reduced by approximately 10% compared to values in fresh samples [17]. These findings show that repeated freezing and

Table 1. Changes of proGRP levels in plasma and serum samples exposed to repeated freeze−thaw cycles.

		proGRP concentrations (pg/mL)			
Sample type	No. of freeze−thaw cycles	Range	Mean (±SD)	Change (%)*	$p^{\dagger}$
Plasma	1	27.6−91.6	41.3 (±14.9)		—
	2	28.4−84.8	40.1 (±13.6)	−2.9	0.006
	3	28.6−85.6	40.5 (±13.9)	−1.9	0.014
	4	26.2−87.8	40.0 (±14.1)	−3.1	0.003
Serum	1	24.3−83.4	36.5 (±13.6)		—
	2	21.9−79.9	34.9 (±13.6)	−4.4	<0.001
	3	19.5−77.2	34.0 (±13.1)	−6.8	<0.001
	4	18.2−77.9	32.8 (±13.5)	−10.1	<0.001

*Percentage change, with a "+" for an increase and a "−" for a decrease relative to baseline (1 cycle); †The p value was calculated using a paired two-tailed t test. proGRP = progastrin-releasing peptide; SD = standard deviation.

Table 2. Thawing time at 37°C and exposure time at room temperature during repeated freezing and thawing of plasma and serum samples.

Sample processing time (min)	Sample type	No. of freeze−thaw cycles			
		1	2	3	4
Thawing time at 37°C	Plasma	2.2	4.2	6.5	8.5
		(0.4)	(0.4)	(0.8)	(0.8)
	Serum	2.3	4.3	6.7	8.7
		(0.8)	(0.8)	(1.0)	(1.0)
Exposure time at room temperature	Plasma	1.3	1.9	2.4	3.2
		(0.2)	(0.6)	(0.8)	(0.9)
	Serum	1.1	1.9	2.2	2.9
		(0.1)	(0.4)	(0.7)	(1.0)

Data are presented as mean ±SD.

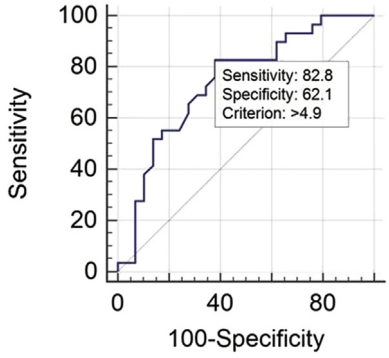

Figure 2. Accuracy of ΔproGRP in discriminating plasma and serum samples that had been exposed to four freeze−thaw cycles, compared with those subjected to one freeze−thaw cycle. ΔproGRP = plasma progastrin-releasing peptide concentration−serum progastrin-releasing peptide concentration.

thawing of samples might facilitate more rapid degradation of proGRP than long-term storage at room temperature.

The degradation rate of proGRP induced by repeated freezing and thawing was greater in serum samples than in plasma samples, consistent with a previous finding [19]. Endogenous proteases such as thrombin are generated in serum during blood coagulation [17]. This might be a cause for the difference of stability between plasma and serum samples. The stability of proGRP was also much higher in plasma samples than in serum samples that were stored for a long time at 4°C or room temperature [17−19]. Taken together, these findings indicate that plasma would be preferred over serum for a diagnostic test or for further research involving circulating proGRP.

We evaluated whether ΔproGRP can be used for detecting repeated freeze−thaw cycles of samples, through AUC analysis. When the cut-off value of ΔproGRP was 4.9, sensitivity and specificity for identifying samples that had undergone four freeze−thaw cycles was 82.8% and 62.1%, respectively. ΔproGRP showed fair accuracy (AUC = 0.741). The assessment accuracy, sensitivity, and specificity of ΔproGRP could be even more elevated when assessing samples exposed to >4 freeze−thaw cycles. Thus, our results demonstrate that ΔproGRP could be used for the assessment of the quality of plasma and serum samples.

Plasma proGRP concentrations ranged from 18.3 to 59.6 pg/mL (median, 40.2 pg/mL) in healthy subjects (aged between 18 and 50) of Chinese Han ethnicity [20]. In other studies that both used healthy Korean subjects, plasma proGRP concentrations were 38.7 ± 14.6 pg/mL (mean ± standard deviation) [19] (in subjects aged between 25 and 52) and 42.5 ± 12.4 pg/mL (in subjects aged between 45 and 83) [9]. Serum proGRP concentrations were found to be 29.4 ± 12.8 pg/mL in healthy Korean subjects (aged between 25 and 52) [19]. These studies have provided reference ranges for proGRP levels in fresh plasma and serum samples from healthy individuals [9,19,20]. In this study, we provide reference ranges for proGRP levels derived from frozen samples.

In conclusion, the stability of plasma and serum proGRP can be affected by multiple freeze−thaw cycles. Thus, biobanks or researchers should minimize the number of freeze−thaw cycles of plasma and serum samples for research using circulating proGRP. ΔproGRP could be used as an indicator to determine whether paired plasma and serum samples have been repeatedly frozen and thawed four times or more.

Conflicts of interest

The authors declare no conflicts of interest.

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