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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