RESEARCH ARTICLE

The influence of the blood handling process on the measurement of circulating TGF-β1

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ABSTRACT. In order to evaluate the impact of blood sample handling processes on circulating TGF-β1 levels, blood specimens were obtained from 13 healthy volunteers using different handling processes (kept at room temperature (RT) or on ice before centrifugation, using different centrifugal forces). TGF-β1 levels were measured using an enzyme-linked immunosorbent assay. A paired-T test was used for statistical analysis. The TGF-β1 level in on-ice serum was significantly lower than that in room-temperature serum (P<0.001), and both were significantly higher than that found in on-ice plasma (P<0.001). Compared with on-ice plasma samples, the longer the samples were kept at RT, the higher the levels of TGF-β1 in plasma (P=0.268, 0.040, and 0.0015 for 5 min, 30 min, and 60 min in RT, respectively). Compared with plasma centrifuged at 2,500×g for 30 min, the TGF-β1 levels were much lower than those found in plasma centrifuged at 1,200×g for 10 min (P=0.003); and a double centrifugation before TGF-β1 detection, significantly decreased the level (P<0.001). It is suggested that the optimal sampling conditions for the detection of TGF-β1 should be plasma prepared on ice and spun down at a higher centrifugal force.

Keywords: transforming growth factor, platelet, plasma, serum

Many factors might influence plasma levels of TGF-β1. In addition to malignancy and radiation damage, which can result in increased TGF-β1 levels [1-4], the reported variability of TGF-β1 levels in human plasma might be a result of the method of preparation of the plasma samples and the assay methodology used. Even when the same type of ELISA was used to measure TGF-β1 levels in normal control subjects, there was still a great deal of variation [5]. It has been reported that apart from being present in plasma, the most abundant source of TGF-β1 in blood is the platelets. There is a 40-100-fold higher level of TGF-β1 in platelets than in any other normal body tissue [6]. Activation or degranulation of platelets during the sample handling process can significantly interfere with TGF-β1 levels in blood samples [4, 7]. However, to the best of our knowledge, there have been no reports published that have evaluated the impact of the actual blood handling process on TGF-β1 levels.

We hypothesized that circulating TGF-β1levels might be being estimated inaccurately as a consequence of platelet contamination/activation during the blood handling process. The purpose of this study was to evaluate the difference in TGF-β1 levels between serum and plasma, the influence of collection conditions, the size of centrifugal force used, and, furthermore, to investigate whether it is possible to rescue a platelet-contaminated plasma sample for measurement of TGF-β1 levels.

METHODS AND MATERIALS

Eligible subjects

This study was approved by the IRB of our institutes. Only healthy adults aged more than 18 years were eligible for this study. Pregnant women were excluded. All subjects with cancer diagnosed within the two years prior to the study, or with any evidence of acute illness were also excluded. All subjects signed a study-specific, written, informed consent.

Blood drawing and sample preparation

Effective venipuncture and blood withdrawal was important for the quality of the samples. Typically, a 20-gauge,

butterfly needle was inserted into an antecubital vein. A tourniquet was used to dilate the vein. The blood was collected into 2 mL vacutainers containing 3.6 mg of K_2EDTA as anticoagulant for preparation of plasma, or into non-additive tubes for the preparation of serum. Samples were mixed by gentle inversion and immediately kept on ice or at room temperature according to the study design. Specifically, six plasma samples were kept at room temperature for 5, 30, and 60 min or on ice for 5, 30, 240 min respectively before centrifugation at $2,500 \times g$ for 30 min (5-min-RT, 30-min-RT, and 60-min-RT plasma; or 5-min-on-ice, 30-min-on-ice, 4-hours-on-ice plasma, respectively); the other plasma sample was kept on ice for 180 min until centrifugation at $1,200 \times g$ for 10 min (low-RCF plasma). Two serum samples were kept at room temperature for 60 min or on ice for 240 min before centrifugation at $2,500 \times g$ for 30 min (RT-serum or on-ice-serum, respectively).

All the centrifugation processes were performed at $4°C$. The upper one third of the plasma or serum supernatant was collected, taking care not to touch the buffy coat, and plasma or serum samples were aliquoted and stored at $-80°C$ until TGF-β1 measurement. Before cytokine detection, a portion of each low-RCF plasma sample was re-centrifuged at $10,000 \times g$ for 15 min (low-RCF-DS plasma).

Detection of TGF-β1

TGF-β1 was measured using an enzyme-linked immunosorbent assay (ELISA). The human TGF-β1 ELISA kits were purchased from R&D system (Quantikine®, Human TGF-β1 Immunoassay. R&D Systems, Inc. Minneapolis, MN, USA). All plasma and serum samples were activated prior to detection using 2.5N acetic acid/10M urea, and were neutralized with 2.7N NaOH/1M HEPES before TGF-β1 detection. Since the plasma or serum TGF-β1 was activated before detection, this procedure cannot distinguish between the active and latent forms of TGF-β1. Therefore, unless otherwise specified, the term "TGF-β1 levels" refers to total TGF-β1.

Statistical analysis

All the data were collected using Microsoft Excel. A paired T test was used to compare different TGF-β1 levels under different conditions. Data were presented with mean ±95% confidence interval unless otherwise specified.

RESULTS

Study populations

Thirteen volunteers were enrolled in this study, two of whom were female and 11 were male. The median age was 30.5 years, ranging from 24 years to 37 years. *Table 1* shows TGF-β1 levels in plasma or serum obtained under different conditions, from the 13 healthy volunteers.

TGF-β1 levels in plasma

Apparently, TGF-β1 levels differ significantly in different plasma samples obtained under different conditions. Waiting time at room temperature is a critical factor for TGF-β1 levels in plasma. Although all spun down at $2,500 \times g$ for 30 min, compared with the TGF-β1 levels in 30-min-on-ice plasma (0.54±0.17 ng/mL), 5 min, 30 min, and 60 min at room temperature could result in the TGF-β1 levels increasing to 1.0±1.39 ng/mL (P=0.268), 2.24±2.78 ng/mL (P=0.040), and 2.75±2.10 ng/mL (P=0.001), respectively. The longer the samples were kept at room temperature, the higher levels of TGF-β1 in the plasma. (*table 1, figure 1*).

Even if kept on ice, when spun down at $2,500 \times g$ for 30 min, a longer waiting time before centrifugation still had small but definite impact on TGF-β1 levels (*table 1, figure 2*). Compared with immediate centrifugation (0.55±0.17 ng/mL), half an hour waiting on ice did not result in a significant change in TGF-β1 levels. However, after a 4-h wait, the TGF-β1 levels increased to 0.69±0.29 ng/mL (P=0.017).

Centrifugal force had a significant impact on plasma TGF-β1 levels (*table 1, figure 3*). Compared with 30-min-on-ice plasma samples spun down at $2,500 \times g$ for 30 min, the 180-min-on-ice plasma samples spun down at $1,200 \times g$ for 10 min showed an increase in TGF-β1 levels from 0.54±0.17 ng/mL to 0.90±0.35 ng/mL (P=0.003). However, if these plasma samples were re-centrifuged at $10,000 \times g$ for 15 min, the TGF-β1 levels could decrease to 0.57±0.15 (P<0.001), which are no longer significantly higher than those found in the 30-min-on-ice plasma (P=0.606) (*table 1, figure 3*).

TGF-β1 levels in serum

The temperature during the waiting interval had a similar impact on TGF-β1 levels in serum. Alhough the waiting time for on-ice serum was much longer in this study (4 h

Table 1
Transforming growth factor-β1 (TGF-β1) levels in plasma and serum prepared under different conditions

| Sample	Temperature	Waiting time (min)	RCF[*] and duration	TGF-β1 (ng/mL)
Plasma	On ice	5	$2,500 \times g$ 30 min	0.55±0.17
	On ice	30	$2,500 \times g$ 30 min	0.54±0.17
	On ice	180	$1,200 \times g$ 10 min	0.90±0.35
	On ice	180	$1,200 \times g$ 10 min then $10,000 \times g$ 15 min	0.57±0.15
	On ice	240	$2,500 \times g$ 30 min	0.69±0.29
	RT	5	$2,500 \times g$ 30 min	1.00±1.39
	RT	30	$2,500 \times g$ 30 min	2.24±2.78
	RT	60	$2,500 \times g$ 30 min	2.75±2.10
Serum	On ice	240	$2,500 \times g$ 30 min	1.27±1.03
	RT	60	$2,500 \times g$ 30 min	19.03±2.49

[*] RCF: relative centrifugal force
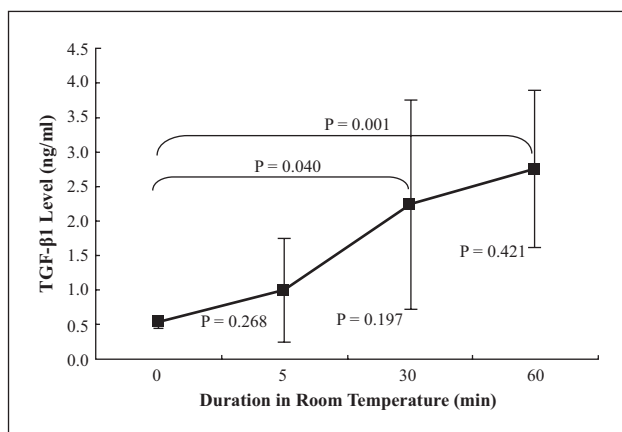
Figure 1
The impact of waiting time at room temperature before centrifugation, on the TGF-β1 levels in plasma (all plasma samples were spun down at 2,500×g for 30 min).
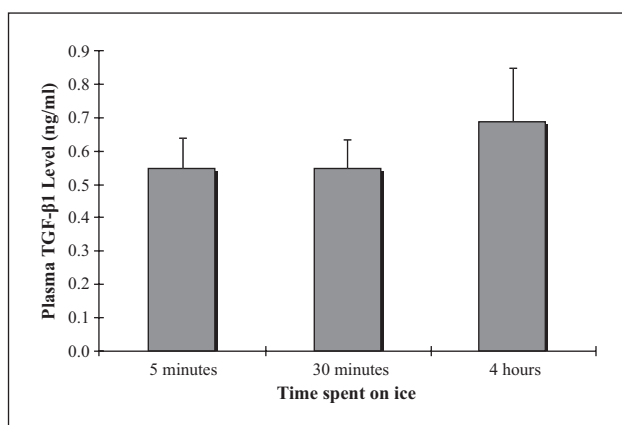

Figure 2
The impact of on-ice waiting time before centrifugation on the TGF-β1 levels in plasma (all the plasma samples were spun down at 2,500×g for 30 min): 5 min *versus* 30 min (P=0.914); 30 min *versus* 4 h (P=0.112); 5 min *versus* 4 h (P=0.017).

on ice *versus* 1 h at room temperature), the TGF-β1 levels were significantly lower than in RT-serum (*P*<0.001), and furthermore, there was no significant correlation between TGF-β1 levels in RT-serum and those found in on-ice serum (P=0.175).
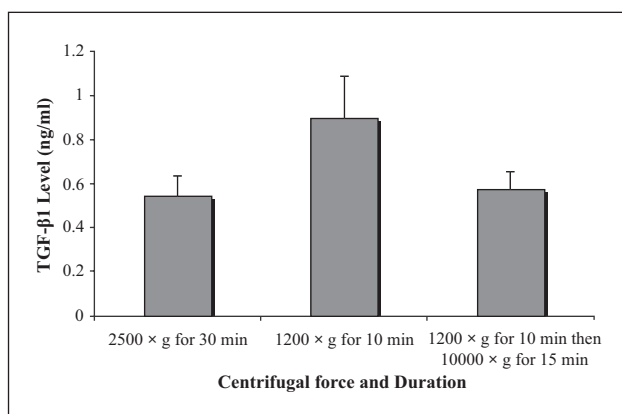

Figure 3
The impact of centrifugal force and double-spinning on the TGF-β1 levels in plasma (all plasma samples were kept on ice before centrifugation): 2,500×g for 30 min *versus* 1,200×g for 10 min (P=0.003); 2,500×g for 30 min *versus* 1,200×g for 10 min then re-centrifugation at 10,000×g for 15 min (P=0.606); 1,200×g for 10 min with *versus* without re-centrifugation (P<0.001).

Differences in TGF-β1 levels between plasma and serum

The TGF-β1 level in RT-serum was significantly higher than in any of the plasma samples (P<0.001 for all plasma samples). However, if the serum was kept on ice before centrifugation, it was no longer significantly higher than 4-hours-on-ice plasma (P=0.066) (*figure 4*), which was similar to 5-min-RT plasma (P=0.574), and was lower than 30-min-RT plasma (P=0.096). Moreover, the TGF-β1 level in on-ice serum was significantly lower than in the 60-min-RT plasma (P=0.013, *figure 4*).

DISCUSSIONS

Our results illustrated that TGF-β1 levels vary with: sample type (serum or plasma), waiting time, temperature, centrifugal force.

Double spinning under very high centrifugal force before TGF-β1 measurement, can normalize the TGF-β1 levels in plasma for plasma samples prepared with a relative low centrifugal force. Temperature also has a critical impact on TGF-β1 levels in serum.

The accurate measurement of circulating TGF-β1 levels is extremely important, as it is a useful marker for many health conditions. TGF-β1 has been known to play a key role in radiation-induced lung toxicity (RILT) [8-12]. Although it has not been reproduced consistently [13-17], it has been reported that plasma TGF-β1 levels at the end of radiotherapy correlated with the later onset of symptomatic lung toxicity in non-small cell lung cancer patients treated with definitive radiotherapy [18, 19]. Numerous factors interfere with the relationship between plasma TGF-β1 levels and the risk of RILT. The accurate detection of TGF-β1 is of primary importance in this relationship. In addition to its important role in radiation pneumonitis, it was also found that TGF-β1 plays an important role in central nervous system, vascular system and respiratory system development [20-22]. TGF-β1 is involved in local signaling for a variety of human diseases including renal diseases, cardiac hypertrophy and fibrosis in heart failure, hepatic fibrosis [23], tumor genesis and progression
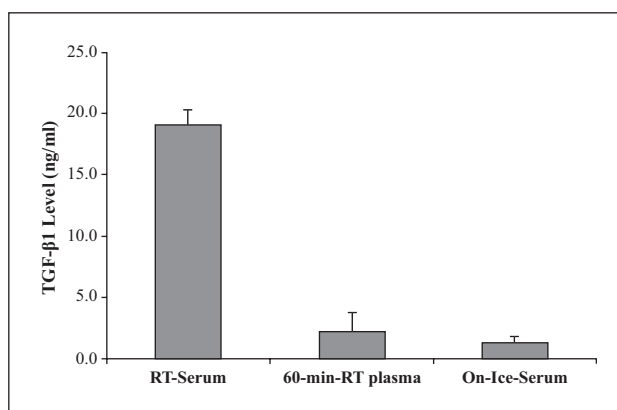

Figure 4
The impact of waiting time at room temperature before centrifugation on the TGF-β1 levels in serum (all the plasma samples were spun down at 2,500×g for 30 min). RT-serum *versus* on-ice serum (P<0.001); RT-serum *versus* 60-min-RT plasma (P<0.001); on-ice serum *versus* 60-min-RT plasma (P=0.013).

[1, 24, 25]. Increased circulating TGF-β1 levels have been reported in many disease conditions [3, 26, 27].

It is well known that platelets have very high TGF-β1 levels, being the largest TGF-β1 reservoir in the body [6]. In serum with well developed clots, all the platelets are activated; the TGF-β1 is released into the supernatant, thus increasing its level, which is consistent with the fact that platelets store high levels of TGF-β1. Thus, using serum as a measure of circulating TGF-β1 levels is inappropriate [7]. Our data showed that for serum prepared at room temperature, in which clotting had occurred, the TGF-β1 level was much higher than in plasma. For serum prepared on ice, in which the clotting process would be delayed by the low temperature, the TGF-β1 level was comparatively lower. This confirms the important impact of platelet activation on TGF-β1 levels.

For the plasma preparation, the blood samples should be placed on ice immediately after collection. However, in clinical practice, this is not always possible. The influence of the time spent at room temperature on TGF-β1 levels has not been fully addressed before. In our study, with the use of anticoagulant, keeping blood samples at room temperature had a remarkable effect on plasma TGF-β1 levels, which again is largely down to platelet activation and hemolysis. Thus, it is very important to keep blood samples on ice or at 4°C immediately after collection.

For anticoagulated blood samples, even if they are kept on ice or at 4°C, a longer time interval before centrifugation still has some influence on the TGF-β1 levels. A four-hour interval for the sample on ice could increase the TGF-β1 level by 25% compared with immediate centrifugation after blood collection. For any biomarker correlation studies, in order to obtain the real, circulating cytokine level, it should be borne in mind that the blood should be handled as soon as possible, even if the blood samples are kept on ice. Otherwise, there could be significant variations in the level of the biomarker.

It is important to note that the centrifugation conditions also have an important influence on TGF-β1 levels. For plasma preparations, the centrifugal force and duration of centrifugation used in different institutes vary significantly, from 700×g for 15 min to 3,600×g for 20 min, sometimes with double spinning [28, 29]. *Table 2* shows the centrifugal force and duration of centrifugation used and the corresponding plasma TGF-β1 levels in normal controls in some selected studies [3,7,26,27,30-34]. High TGF-β1 levels were seen in all samples centrifuged with a centrifugal force and duration of less than 3,000×g for 30 min. Using blood samples from the same healthy donor, we evaluated the impact of different centrifugal forces on TGF-β1 levels. Our results showed that a lower centrifugal force gave significantly higher TGF-β1 levels: 1,200×g for 10 min is not enough to remove platelets from plasma. It is recommended that at least a centrifugal force of 2,500×g for 30 min be used to ensure the removal of platelets from the plasma. For plasma samples centrifuged at a relatively low centrifugal force, double spinning at 10,000×g for 15 min could improve the quality of the samples significantly.

In addition to TGF-β levels, the process of blood handling might also impact on many other cytokines or chemokines [35]. It was found that the number of platelets correlated significantly to vascular endothelium growth factor (VEGF) concentrations in serum (p=0.002), but not to VEGF concentrations in plasma, and that VEGF concentrations in serum increased with increasing clotting time until a plateau was reached between 2 and 6 h of *in vitro* clotting [36, 37]. VEGF concentrations are also significantly different in plasma samples prepared with different anticoagulants. Plasma where sodium citrate was used as the anticoagulant had significantly higher levels of VEGF than plasma with EDTA as the anticoagulant [38-40]. The variation in blood sample preparation may contribute to the inconsistency in the clinical relevance of VEGF in patients with malignant disease. Platelet activation leads to the release of α-granule chemokines, including CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL17, CXCL1 (growth-regulated oncogene-alpha), CXCL5 (ENA-78), and CXCL8 (IL-8), which attract leukocytes, and further activate other platelets [41]. Since blood handling processes inevitably induce platelet activation to some degree, the clinical implication of this should to be taken into consideration, as well as its impact in correlative studies.

Table 2
Different sample preparation methods and transforming growth factor-β1 (TGF-β1) levels in the literature

Authors	Publication date	Anticoagulant	Relative centrifugal force	Capture and detecting antibody	Mean value (ng/mL) ± standard deviation
van Waarde [30]	1997	K3-EDTA	1,000×g 30 min	Genzyme	9.3±2.1
Grainger [31]	1995	Trisodium citrate	250×g 15 min, then 700×g 15 min	R&D system	9.73± 4.43
Pfeiffer [27]	1996	EDTA	800×g 10 min, then 2,500×g 15 min, then 3600×g 20 min	R&D system	3.1±0.4
Jeon [7]	2001	Potassium-EDTA	1,000×g 20 min, then 3000×g 10 min	R&D system	0.98±0.26
Coupes [32]	2001	EDTA	1,200×g 10 min	Genzyme and R&D system	7.7 (median)
Kong [3]	1995	EDTA	3000×g 20 min	Genentech Inc	4.3±0.3
Junker [26]	1996	Lithium-heparin	800×g 10 min, then 2700×g 10 min	NA	3.8±2.9
Esmatjes [33]	2001	EDTA	1,000×g 30 min	R&D system	3.0±1.1
Wakefield [34]	1995	Sodium-EDTA	1,200×g 30 min	NA	4.3±1.4

In summary, serum and plasma TGF-β1 levels differ significantly, both blood sampling conditions and centrifugation conditions have a great influence on the TGF-β1 level. In some of the published literature, circulating TGF-β1 levels may be inaccurately estimated as a result of platelet contamination/activation during the sampling process. In order to avoid platelet-derived, TGF-β1 contamination, the optimal method for measuring circulating TGF-β1 should be to use plasma prepared on ice and spun down at higher centrifugal forces.

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