Dependence of TIMP-1 Plasma Levels on Preanalytical Specimen Handling

Marie Dresse a Dorothea Nagel a Eva-Maria Ganser a Gerard Davis b Barry Dowell b Robert Doss b Petra Stieber a

a Institute of Clinical Chemistry, Klinikum Grosshadern, University of Munich, Munich, Germany; b Abbott Diagnostic Division, Chicago, Ill., USA

Introduction

Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a 28-kDa glycoprotein which can be measured in peripheral blood by immunoassay [1]. The clinical use of TIMP-1 in various diseases is currently being investigated and has been discussed by several groups. Several studies indicate that TIMP-1 may have clinical utility as tumor marker, since elevated levels occur in many patients suffering from colorectal cancer compared to healthy individuals [2–4]. Elevated TIMP-1 levels in colorectal cancer patients correlate with poorer prognosis and response to chemotherapy [5, 6]. Elevated TIMP-1 levels have also been shown to predict worse outcome in breast cancer patients [7]. Furthermore, TIMP-1 plasma levels seem to play an important role in metabolic disorders, such as diabetes and adiposity [8–10], as well as in patients with cardiac disease [11–15] and sepsis [16]. In addition to these promising investigations, some authors have presented results indicating that TIMP-1 levels differ depending on pre- and perianalytical conditions [17–22]. Higher levels of TIMP-1 have been measured in serum than in plasma, which raised suspicion that TIMP-1 is released by leukocytes and platelets during the coagulation process and therefore plasma has been suggested to be the appropriate medium to measure TIMP-1 levels in peripheral blood [17, 19–21]. But even in plasma, changes in TIMP-1 levels under different specimen handling conditions have been observed [19, 21, 22]. During our own investigations of TIMP-1 as a possible tumor marker, we...
measured higher TIMP-1 levels in the plasma specimens of healthy persons than in those of patients suffering from colorectal cancer, which may have been caused by the fact that the plasma specimens of the 2 groups had not been handled identically prior to their measurement. However, our results could not be explained by the existing studies on this topic. The present study was performed to verify our own observations and to understand how TIMP-1 levels in plasma are influenced by the preanalytical conditions of blood specimens that they would likely to be exposed to in daily routine, such as time to centrifugation, exposure to light, mechanical manipulation and freezing/thawing.

Materials and Methods

Sampling
Blood was collected from the antecubital vein of 20 healthy volunteers using a tourniquet. All specimens were simultaneously taken in plastic tubes of 2.3 ml capacity containing EDTA (Monovette Systems; Sarstedt AG & Co., Nümbrecht, Germany).

Experimental Setup
The pattern of the trial is shown in figure 1. From each of the 20 participants, 26 blood specimens were extracted into individual tubes. Two of each of the 26 specimens were processed into plasma and TIMP-1 was measured within 1 h after venipuncture. The remaining plasmas were kept frozen at −80°C. One of the 2 specimens from each person was then thawed and measured again after 24 h and refrozen, before both were thawed and measured after 72 h. The other 24 blood specimens from each person were stored under different conditions prior to their centrifugation and measurement: 8 were kept at 20°C and exposed to daylight, 8 were kept at 20°C but covered with aluminum foil and 8 were stored at 4°C in the cold storage room, where they were also exposed to light. The impact of mechanical manipulation on the blood specimens was tested by mixing 4 of each of these 8 specimens from all 3 storage conditions on a turntable for 30 min per day until centrifugation. A mixed and an unmixed specimen of each group was then centrifuged and measured after 3, 6, 24 and 72 h.

Laboratory Methods
All specimens were centrifuged at 2,000 g at 4°C for 10 min. The supernatant was transferred into dry plastic tubes. TIMP-1 was measured using an automated immunoassay (Architect System; Abbott Diagnostics, Abbott Park, Ill., USA). To test interassay variation, 2 specimens of each of 3 controls with low, intermediate and high TIMP-1 concentrations were simultaneously measured in each run. The mean (with SD in parentheses) and median (with range in parentheses) TIMP-1 values were 51.1 ng/ml (1.2) and 51.29 ng/ml (48.4–53.1) for the low controls, 103.0 ng/ml (2.4) and 102.7 ng/ml (98.8–108.4) for the medium controls and 312.5 ng/ml (9.9) and 312.5 ng/ml (294.2–333.2) for the high controls. The coefficients of variation resulting from these data are 2.4%, 2.3% and 3.2%, respectively.

Statistics
All statistical calculations were done using SAS (version 9.1; SAS Institute, Cary, N.C., USA). For comparisons of TIMP-1 levels in corresponding plasma specimens, a t test was performed. Results were considered significant for p < 0.05.

Results
Freezing/Thawing
It was found that TIMP-1 levels increased after each freeze/thaw cycle. The measured TIMP-1 levels and the differences between the thawed specimens and those measured directly after centrifugation are shown in figure 2. Significantly higher TIMP-1 values were measured in specimen 1 after its second freeze/thaw cycle 72 h after venipuncture than after the first freeze/thaw cycle 24 h after venipuncture (p < 0.0001). In contrast, TIMP-1 values in specimen 2 measured after its first thawing 72 h after venipuncture were not higher than those in specimen 1 measured after its first thawing 24 h after venipuncture (p = 0.71).

Mechanical Manipulation
While the applied mixing procedure did not influence TIMP-1 levels in the specimens centrifuged and measured after 3 and 6 h, the mixed specimens stored 24 and 72 h prior to centrifugation showed significantly higher TIMP-1 levels than the corresponding unmixed specimens (p < 0.001). According to the experimental setup,
the specimens centrifuged after a storage time of 24 or 72 h had been put on the turntable 2 and 3 times, respectively, while those centrifuged after 3 and 6 h had only been mixed once. The differences found between mixed and unmixed specimens are shown in figure 3. As the differences were similar under all storage conditions, all specimens were evaluated combined irrespective of where they had been kept prior to centrifugation.

**Storage Time and Conditions**

To simplify the evaluation of the impact of storage time and conditions prior to centrifugation on TIMP-1 levels, only the specimens which had not been mixed were considered. It was found that in all these specimens, TIMP-1 levels were higher than in the specimens centrifuged and measured directly after venipuncture. Corresponding data are shown in table 1 and figure 4. The increase was significant after all tested time intervals of 3, 6, 24 and 72 h, the extent of the increase, however, varied depending on the storage conditions. TIMP-1 levels increased significantly more in the specimens stored at 20°C than in those stored at 4°C and the increase was significantly higher in the specimens exposed to light than in the covered ones. Results are shown in figure 4.

**Discussion**

Many results have been published indicating that TIMP-1 may have clinical utility as a biomarker in colorectal cancer [2–6] and in other diseases [7–16]. Several studies have shown that TIMP-1 blood levels are dependent on preanalytical conditions [17–22]. During our own investigations on the clinical use of TIMP-1 we made some unexpected observations that likely could only be explained by a possible bias of different specimen handling used. Since the existing data on this topic could not explain our observations adequately, we performed the present study. In contrast to the results presented by Holten-Andersen et al. [19] and Alby et al. [21], we measured increased TIMP-1 levels after each freezing and thawing cycle, which is in accord with our previous observations [unpubl. data] and which indicates that the increase in
TIMP-1 is not dependent on the storage time at –80°C, but is caused by the freezing/thawing procedure itself. While Alby et al. [21] used heparin plasma instead of EDTA plasma and a different centrifugation mode, Holten-Andersen et al. [19] performed their freeze/thaw study similar to our trial. So even if the reason for these different findings cannot be found, it has to be stated that studies on the clinical use of TIMP-1 should only be performed using plasma specimens which are comparable regarding their preanalytical factors, such as storage time and number of previous freeze/thaws. Our results concerning specimen handling prior to centrifugation clearly show that TIMP-1 increases when whole blood specimens are stored prior to their further processing into plasma. This phenomenon can possibly be explained by the degradation of platelets in these blood specimens, as platelets contain high concentrations of TIMP-1 as reported by Cooper et al. [23]. While in our trial the observed increase was significant even after a storage time of 3 h, Holten-Andersen et al. [19] only obtained a significant increase after a storage time of 8 h in their similar trial. We cannot confirm that reliable TIMP-1 values can be measured when blood specimens are centrifuged within 8 h. Our findings rather suggest that the increase is a steady process and that blood specimens have to be centrifuged and measured immediately after venipunc-

**Fig. 3.** Mechanical manipulation: TIMP-1 levels in all mixed specimens compared to those measured in the unmixed specimens after all tested time intervals.

**Table 1.** Time to centrifugation

<table>
<thead>
<tr>
<th></th>
<th>Mean difference of TIMP-1 levels to directly measured specimens after 3 h</th>
<th>after 6 h</th>
<th>after 24 h</th>
<th>after 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage at 20°C, daylight</td>
<td>21.0 (12.5)</td>
<td>28.0 (13.1)</td>
<td>38.5 (13.0)</td>
<td>45.9 (15.1)</td>
</tr>
<tr>
<td>Storage at 20°C, covered</td>
<td>10.0 (9.8)</td>
<td>18.7 (14.5)</td>
<td>28.7 (14.5)</td>
<td>39.1 (14.3)</td>
</tr>
<tr>
<td>Storage at 4°C, daylight</td>
<td>7.7 (6.7)</td>
<td>11.7 (13.3)</td>
<td>14.5 (13.1)</td>
<td>23.8 (12.1)</td>
</tr>
</tbody>
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Figures in parentheses are SD. The differences found between TIMP-1 values in the specimens stored before centrifugation and those centrifuged directly after blood collection for all time intervals and storage conditions are shown. p < 0.001 for all obtained results.
ture in order to obtain reliable TIMP-1 values. The lack of significance after 8 h in the study of Holten-Andersen et al. [19] might be caused by the relatively low number of blood donors: they only used 8 blood donors, while in our trial 20 persons were involved. Furthermore, the automated prototype Architect immunoassay for TIMP-1 used in our trial provides more precise and reliable data than the manual ELISA kits used so far, especially when specimens have to be measured quickly and in several runs per day as necessary for the investigation of preanalytical biases. Additionally, we investigated the impact of storage temperature and exposure to light on TIMP-1 levels. According to the findings of Lomholt et al. [22], storage of the whole blood specimens in the cold storage room at 4°C could reduce the increase in TIMP-1. Similarly, covering the testing tubes with aluminum foil to protect them from light had the same effect, even if to a lesser extent. These results lead to the conclusion that blood specimens used for the measurement of TIMP-1 should be kept covered and refrigerated for as short as possible prior to their centrifugation and measurement, which can hardly be realized in daily clinical routine; as TIMP-1 levels had already increased after a storage time of 3 h, which was the shortest time interval tested, no definite recommendation regarding a maximum storage time can be given. We furthermore tested the impact of mechanical manipulation on the whole blood specimens as they might be exposed to when they have to be sent by post to the laboratory. A significant increase in TIMP-1 values could only be found in the specimens mixed twice or more after 24 h or later with most of them being macroscopically hemolytic. This indicates that any commonly used transportation of the specimens can be performed within 24 h without causing an increase in TIMP-1. However, considering the fact that TIMP-1 values steadily increase when whole blood is stored prior to centrifugation, it has to be suggested that transportation of specimens should preferably be performed after processing them into plasma and placing them in secondary tubes.

The present study underlines the importance of a careful investigation of the stability of new biomarkers and the need for specimen handling standards in order to obtain reliable data.

**Acknowledgement**

The authors thank laboratory technician Cornelia Scheuer for her skillful and reliable laboratory work.
References


