found seasonal variation [27], and simple and noninvasive sample collection. In addition to 24-h samples, as used in this study, OC can be measured from spot urine samples collected as the first or second morning void [9,11]. Moreover, U-OC responds to therapy more rapidly than S-OC.

In summary, the results suggest that U-OC is a useful marker for monitoring short-term changes in bone metabolism in response to antiresorptive therapy in postmenopausal women.

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References


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Effect of Specimen Anticoagulation on the Measurement of Circulating Platelet-Derived Growth Factors, Robert Zimmermann, Julia Koenig, Juergen Zingssem, Volker Weisbach, Erwin Strasser, Juergen Ringwald, and Reinhold Eckstein (Department of Transfusion Medicine and Hemo-
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Platelets (PLTs) contain an assortment of growth factors (GFs), in particular PLT-derived GFs (PDGFs), transforming GFs (TGFs), and vascular endothelial GF (VEGF) (1). By topical release and action of these GFs simultaneously with thrombus formation and bleeding cessation, PLTs initiate the processes of wound repair, angiogenesis, and defense against infectious agents (1). Released GFs may also have distant effects if they reach the fluid compartment of the circulating blood. Increased serum PDGF concentrations during and after hemodialysis, attributable to PLT-membrane contact at artificial surfaces, may be directly involved in the increased frequency of atherosclerosis in hemodialysis patients (2). The role of circulating GFs in many other nonmalignant clinical conditions has also been investigated (3–8).

Increased concentrations of VEGF have been found in a wide spectrum of malignancies (9–16). Many researchers assume a prognostic relevance of this finding because
VEGF is crucial in angiogenesis, which in turn is crucial for metastasis and tumor growth (9, 11). The question of whether serum or plasma is the best specimen to use for the measurement of circulating VEGF has not been answered. Increased VEGF concentrations in cancer are detectable in serum and plasma. The concentrations are much higher in serum than in plasma, however, because VEGF is released from PLTs in vitro during the clotting process (9, 11, 12). Nevertheless, many researchers prefer to measure serum VEGF because combined cellular and soluble GFs act at the tumor site (12, 13, 15). Furthermore, PLTs may act as scavengers for tumor-derived GFs (10, 15, 16).

Although EDTA may activate PLTs (17, 18), released GFs reportedly can be measured in EDTA plasma as well as in citrate plasma (11), and there are clinical reports of GF measurements from EDTA plasma (8, 19, 20). There are also differences in the degree of PLT activation by different anticoagulants that contain citrate (21, 22).

We performed comparative measurements of 4 GFs that originated from PLTs and examined 3 samples of differently anticoagulated plasma and serum from 30 healthy volunteer blood donors (23 male, 7 female). The study was approved by the Institutional Ethics Committee of the University Erlangen-Nuremberg. All donors met the relevant guidelines (23, 24). All specimens were collected into plastic tubes (Sarstedt).

Analyses of cell concentrations, PLT CD62 expression, and PLT in vitro aggregability were made from citrate-theophylline-dipyridamole-adenosine (CTAD) whole-blood specimens (CTAD-to-blood ratio, 1:9 by volume), and tripotassium EDTA whole-blood specimens (1.6 mg of EDTA per 1 mL of blood). PLTs, leukocytes, granulocytes, lymphocytes, and monocytes were counted with a cell counter (Advia 120 Automated Hematology System; Bayer Corporation). Activated PLTs were measured by their spontaneous expression of CD62 (P-selectin, GMP-140) within 120 min after sampling. In addition, PLT response to activating agents was tested by their CD62 expression after activation by ADP (final concentration, 20 μmol/L). We analyzed samples in a flow cytometer (FACSCalibur; Becton Dickinson) with software. The results are reported as percentages of specific CD62+ cells. After adjusting the PLT concentration to 250 × 10^6/L with autologous plasma, we measured PLT aggregation with an aggregometer (PAP 4; Bio-Data) and with activating agents at the following final concentrations: 20 μmol/L ADP, 1.2 g/L ristocetin, and 0.19 g/L collagen. The aggregation response was calculated with the light transmission of PLT-rich plasma set as 0% and the light transmission of PLT-poor plasma set as 100%. The final extent of aggregation was measured 2 min after addition of the aggregating agents.

The concentrations of PDGF-AB, TGF-β1, VEGF, and basic fibroblast GF (bFGF) were measured in the supernatants of the 3 anticoagulated specimens and in serum by sensitive and specific immunoassays according to the manufacturer’s instructions (R&D Systems). The detection thresholds (CVs) provided by the manufacturer were 6 ng/L (8.5%) for PDGF-AB, 7 ng/L (12.8%) for TGF-β1, 9 ng/L (8.8%) for VEGF, and 3 ng/L (9.1%) for bFGF. Before analysis of TGF-β1, acid activation and neutralization were performed to activate latent TGF-β1 to the immunoreactive form. Calibrators and samples were assayed in duplicate, and mean values were calculated. In the diluted samples for TGF-β1 analysis, the results were multiplied by the appropriate factor.

We analyzed data with statistical software (SPSS for Windows, release 11.5.1; SPSS). Results were tested for gaussian distribution by the Lilliefors and the Shapiro-Wilks tests. For results that were not gaussian-distributed, analysis was performed with the nonparametric Mann-Whitney U-test, and for results that were gaussian-distributed, a t-test for paired or unpaired data was used when appropriate. Linear associations between 2 variables were ascertained with the Pearson r test. P < 0.01 was considered a significant correlation.

The results for all measurements of cell counts, CD62 expression, in vitro aggregability, and soluble GFs are summarized in Table 1. In blood anticoagulated with CTAD, spontaneous CD62 expression and PLT activation by ADP were both low. In addition, CTAD plasma contained the lowest mean concentrations of all measured GFs. The bFGF concentration in CTAD plasma correlated weakly to the absolute number of lymphocytes per milliliter in CTAD blood (P = 0.001; r^2 = 0.224).

The in vitro activation of PLTs in citrate blood was only slightly higher than in CTAD plasma, but the aggregability by ADP was not blocked as by CTAD. The mean concentrations of PDGF-AB and TGF-β1 in citrate plasma were higher than in CTAD plasma (P < 0.01). Despite the small magnitude of this difference, the number of activated CD62+ PLTs per milliliter in citrate blood was correlated with the PDGF-AB concentration (P = 0.001; r^2 = 0.348).

Use of EDTA as the anticoagulant led to pronounced in vitro PLT activation in some samples and to minor activation in others. The additional PLT aggregation responses to ADP, epinephrine, ristocetin, and collagen were severely impaired by EDTA. Interestingly, there was a strong correlation (P < 0.001) between the absolute number of activated, CD62+ PLTs per milliliter and the concentrations of the GFs PDGF-AB (r^2 = 0.694), TGF-β1 (r^2 = 0.901), and VEGF (r^2 = 0.513; Fig. 1).

Serum contained much higher concentrations of the GFs PDGF-AB, TGF-β1, VEGF, and bFGF than all anticoagulated specimens (P < 0.01). There was a weak correlation between the PDGF-AB concentrations in serum and the number of circulating monocytes per milliliter (P = 0.007; r^2 = 0.250).

These results demonstrate that CTAD is the only anticoagulant that completely blocks the in vitro release of GFs from PLTs. Sodium citrate is not as effective or reliable. The results of our measurements of GFs in EDTA plasma demonstrate that the extent of in vitro PLT activation by EDTA with subsequent release of GFs shows an
unexpectedly wide interindividual range. In addition, there was a strong correlation between the amount of individual PLT activation by EDTA and the concentrations of released GFs in EDTA plasma. Like VEGF concentrations, PDGF-AB and TGF-β1 concentrations were much higher in serum than in plasma. Nevertheless, it is doubtful that the clotting process releases all PLT-derived GFs into the serum. Recently we showed that PLT destruction by the detergent Triton X-100 causes the release of much more GFs from PLTs into the supernatant than do in vitro clotting processes (25). In addition, Tezono et al. (26) recently demonstrated that VEGF is partially trapped within fibrin clots. Of course, the trapped GF fraction cannot be detected in the serum. Finally, the correlation between GF concentrations and the number of certain leukocytes in some specimens confirms previous findings (25). However, the majority of reports on serum or plasma concentrations of VEGF give no information on the leukocyte content in the examined samples.

In conclusion, for measurement of circulating GFs originating from PLTs, we recommend the use of CTAD plasma only for detection of molecules that have been released in vivo. To measure soluble and cellular GFs that are contained in PLTs, we propose analysis from whole-blood specimens after Triton X-100 lysis of PLTs. EDTA plasma should not be used to measure circulating PLT-derived GFs because of the extreme interindividual vari-

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Table 1. Blood cell concentrations, PLT activation markers, PLT aggregability, and concentrations of PDGF-AB, TGF-β1, VEGF, and bFGF in CTAD plasma, citrate plasma, EDTA plasma, and serum.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Specimen anticoagulated with</th>
<th>Mean (SE) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTAD</td>
<td>EDTA</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>Citrate</td>
</tr>
<tr>
<td>Leukocyte concentration, 10⁹/L</td>
<td>4.7 (0.25)</td>
<td>4.7 (0.25)</td>
</tr>
<tr>
<td>PLT concentration, 10⁹/L</td>
<td>207.0 (7.0)</td>
<td>189.2 (5.8)</td>
</tr>
<tr>
<td>Spontaneous CD62 expression, % positive PLTs</td>
<td>0.6 (0.1)</td>
<td>1.1 (0.1)</td>
</tr>
<tr>
<td>Additional CD62 expression induced by 20 μmol/L ADP, % positive PLTs</td>
<td>1.2 (0.4)</td>
<td>30.1 (2.7)</td>
</tr>
<tr>
<td>PLT aggregation, % light transmission</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 μmol/L ADP added</td>
<td>3.6 (0.3)</td>
<td>71.3 (3.9)</td>
</tr>
<tr>
<td>1.2 g/L ristocetin added</td>
<td>52.7 (3.3)</td>
<td>86.2 (3.0)</td>
</tr>
<tr>
<td>0.19 g/L collagen added</td>
<td>48.5 (3.7)</td>
<td>81.0 (1.7)</td>
</tr>
<tr>
<td>PDGF-AB, ng/L</td>
<td>8.9 (2.0)</td>
<td>22.5 (2.1)</td>
</tr>
<tr>
<td>TGF-β1, μg/L</td>
<td>2.6 (0.4)</td>
<td>4.4 (0.3)</td>
</tr>
<tr>
<td>VEGF, ng/L</td>
<td>19.2 (2.8)</td>
<td>21.5 (2.5)</td>
</tr>
<tr>
<td>bFGF, ng/L</td>
<td>0.27 (0.1)</td>
<td>0.29 (0.1)</td>
</tr>
</tbody>
</table>

a,b Data distribution: a gaussian; b nongaussian.
c P < 0.01 vs values in EDTA plasma.
d P < 0.01 vs values in citrate plasma.
e P < 0.01 vs values in CTAD plasma.
f P < 0.01 vs values in serum.

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Fig. 1. Scatter diagrams showing the correlation between CD62+ PLTs per milliliter and the concentrations of PDGF-AB, TGF-β1, and VEGF in EDTA plasma.
ation of PLT activation and concurrent in vitro GF release by EDTA.

References

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