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Hematopoietic growth factors

Administration of granulocyte-colony-stimulating factor for allogeneic hematopoietic cell collection may induce the tissue factor-dependent pathway in healthy donors

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Summary:

The hypercoagulable state caused by the use of recombinant human granulocyte colony-stimulating factor (rhG-CSF) has been cited in anecdotal reports. Since tissue factor (TF) is the main initiator of the coagulation cascade, we examined if rhG-CSF had an inductive effect on the TF-dependent pathway in 18 healthy donors receiving rhG-CSF (10 μg/kg/day $\times$ 5 days) for peripheral blood progenitor cell mobilization. After rhG-CSF, there were increases both in TF antigen (TF:Ag) ($P = 0.01$) and TF procoagulant activity (TF:PCA) ($P = 0.06$) plasma levels and in TF:Ag cytofluorimetric expression on CD33 (+) cells ($P = 0.04$). Mean activities of FVIII and vWF also increased significantly. Thrombin time was slightly prolonged ($P = 0.06$) due to significant increases in plasma D-dimer levels. In addition, while FIX activity remained stable, there were marked reductions in mean plasma FX and FII activities and a slight decrease in FVII activity that resulted in a significant prolongation of prothrombin time within normal ranges. In conclusion, the administration of rhG-CSF led to a 'prothrombotic state' via stimulation of TF and increased endothelial markers, such as F VIII and vWF. In the light of these findings, the use of rhG-CSF for stem cell mobilization should be undertaken cautiously in healthy donors with underlying thrombotic risk factors.

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Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is a hematopoietic cytokine that stimulates neutrophil production and release of mature granulocytes from bone marrow and enhances neutrophil function. Owing to its capability to mobilize $CD34^+$ hematopoietic progenitor cells (HPC) into circulating blood, rhG-CSF use has been established for mobilization and collection of HPC for both allogeneic and autologous transplantations. The most commonly used protocol for hematopoietic cell mobilization is the administration of rhG-CSF to donors at a moderate–high dose such as 10–12μg/kg/day for 4 or 5 days and subsequent leukopheresis.[1]

The safety of rhG-CSF administration in healthy donors has been investigated in several studies.[1,2] However, there are limited cumulative data about the effects of rhG-CSF on hemostasis. Thrombosis was the first adverse event attributable to rhG-CSF,[3] and two cases of healthy donors with acute arterial thrombosis, which might be related to rhG-CSF, have been documented.[1] In a meta-analysis of studies investigating the use of rhG-CSF with chemotherapy, 1.2% of cancer patients experienced thrombotic complications.[4] Whether or not rhG-CSF has a direct role in thrombus formation is still a mystery. Following preliminary studies that showed the presence of a receptor for rhG-CSF on platelets[5] and megakaryocytes[6], it was claimed that rhG-CSF caused thrombosis by increasing platelet aggregation.[7] TF is the main physiological initiator of the blood coagulation-protease cascade in normal hemostasis. It has a cofactor role for factor VII/factor VIIa (FVII/FVIIa) during activation of factor IX (FIX) and factor X (FX) by FVII.[8]

In physiological conditions, peripheral blood cells (monocytes/macrophages) and endothelial cells do not express TF.[9] However, recent studies have shown that monocytes and macrophages also contributed to thrombin generation under various pathological conditions such as Gram-negative sepsis, endocarditis, trauma, chronic inflammation and unstable angina.[10,11]

Currently, there is a lack of information about the role of TF in hypercoagulable state induced by the use of rhG-CSF for allogeneic HPC mobilization in healthy donors. This study aimed to show the contribution of rhG-CSF to plasma tissue factor antigen (TF:Ag) and tissue factor procoagulant activity (TF:PCA) as well as cell surface TF:Ag cytofluorimetric analysis on peripheral blood cells, factors involved in intrinsic and extrinsic coagulation pathways, natural anticoagulants and contribution of endothelium-derived factors in healthy donors.

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Patients and methods

A total of 18 HLA-identical healthy sibling donors who were candidates for allogeneic stem cell collection (eight female donors, 10 male donors; median age 30 years, range 22–48 years) were studied. After obtaining written consent, donors received 5 or 6 μg/kg rhG-CSF (Filgrastim; Amgen, Roche) subcutaneously twice daily for nine consecutive doses. Following the ninth dose on day 5, leukapheresis was performed using a continuous-flow cell separator (COBE Spectra, COBE BCT Inc., Lakewood, CO, USA or Baxter CS 3000 plus, Baxter Healthcare System, Deerfield, IL, USA), processing two times the total blood volume of the donor with the aim of collecting 4×10^6/$CD34^+$ cells/kg recipient weight. The donors were routinely surveyed for thrombotic events during rhG-CSF administration, collection and in the follow-up period for a median of 18 months (range 12–25).

Blood collection

Blood (35 ml) was drawn from each donor before the first dose (day 0) and after the ninth dose of rhG-CSF (day 5). EDTA anticoagulated tubes (citrated Na 0.105 M; BD, USA) were used for complete blood count (CBC), differential leukocyte counts and detection of TF:Ag antigen expression. Citrated (3.8% trisodium citrate anticoagulant solution; BD, USA) tubes were used for coagulation tests and for the study of TF:Ag and TF:PCA. Plasma samples obtained by centrifugation of citrated tubes at room temperature (3000 *g*, 10 min) were processed immediately or stored at -80°C as aliquots.

Standard hematological and hemostatic parameters

CBC and white blood cell (WBC) differential counts were obtained by processing anticoagulated blood specimens through a properly calibrated and external quality-controlled automated hematology analyzer (STKS, Beckman Coulter, USA).

Coagulation parameters including prothrombin time (PT) (normal range 11–15 s), partial thromboplastin time (PTT) (normal range 24–36 s), thrombin time (TT) (normal range 12–16 s), factor II (FII), FVII, FVIII, FIX, FX (normal range 50–150%) and plasma fibrinogen levels (normal range 200–400 mg/dl) were measured nephelometrically in an automated and external quality-controlled coagulometer (ACL, Futura, IL, Milan, Italy) using reagents purchased by the same manufacturer. The level of D-dimer was measured using a latex-enhanced turbidometric immunoassay in the same coagulometer (IL, Milan, Italy). vWF Ri:CoF activity (normal range 50–150%) was measured semiquantitatively using von Willebrand reagent (Dade Behring, Marburg, Germany). Natural anticoagulants including protein C, protein S and antithrombin III (ATIII) (normal ranges 70–140, 60–140, 75–122%, respectively) were measured functionally (IL Test Antithrombin, IL test Protein C and IL test Protein S, Milan Italy). Activated protein C resistance (APC) ratio (normal range 2–3.5) was measured by IL modified APC-R test kit (IL, Milan, Italy) using factor V-deficient plasma.

Plasma TF antigen and PCA assays

Plasma TF:Ag. Plasma TF:Ag was determined using an 'Imubind Tissue Factor' ELISA kit (#845, American Diagnostic, Greenwich, USA). The kit employs a murine anti-human TF monoclonal antibody for antigen capture. Prediluted plasma samples (1/10 in sample buffer) were incubated in micro-test wells precoated with the capture antibody. Captured TF was detected with a biotinylated antibody fragment that specifically recognizes bound TF. TF levels were determined by measuring absorbency at 405 nm and by comparing values with those of standard curve.

Plasma TF:PCA. Plasma TF:PCA was determined using an Actichrome Tissue Factor Activity Kit (#846, American Diagnostic, Greenwich, USA), which measures intact TF as well as TF/FVII and TF/FVIIa complexes. Peptidyl activity of TF present in plasma was measured to form a complex with FVII. Plasma samples diluted 1/10 with 5% TF/Tissue Factor Pathway Inhibitor depleted plasma, mixed with human FVIIa and incubated, allowing formation of the TF/FVII complex. The complex was allosterically activated, and its activity was directly measured by its ability to cleave to a highly specific chromogenic substrate for TF/FVIIa complexes (Spectrozyme FVIIa) that was added to the reaction solution. Releasing a paranitroaniline chromophore into the reaction solution terminated the cleavage of the substrate. Absorbency of the solution was read at 450 nm and compared with the values obtained from a standard curve generated using known amounts of lipidated TF. The results were multiplied by 10 (dilution factor) and 42, conversion factor for TF:PCA, as per the recommendation of the manufacturer (American Diagnostica, Greenwich, USA).

Cell surface TF antigen cytofluorimetric analysis

TF:Ag expression was detected using a fluorescein isothiocyanate (FITC)-conjugated murine monoclonal antibody (moAb) specific to human TF (#4508CJ, American Diagnostica, Greenwich, USA). Whole blood was diluted with PBS to obtain a dilution of 0.5×10^6 WBC/ml. In order to identify specific binding to monocyte/neutrophils, monoclonal antibodies specific to CD14-phycoerythrin (PE), CD45-peridinin chlorophyll (PerCP) and $CD33^{PE}$ and an isotypic control (IgG1-irrelevant monoclonal antibodies, that is, FITC-labeled clone, PE-labeled clone and PerCP-labeled clone, respectively) were used simultaneously (Becton Dickinson Immunologic System, BDIS, San Jose, USA). In all, 5 μl of anti-TF moAb and 7 μl of other moAbs were added to prelabeled tubes. Monoclonal antibodies were incubated with 100 μl diluted blood sample for 30 min at 4°C. Following red cell lysis procedure, cells were washed twice with PBS and fixed with 500 μl 1% paraformaldehyde solution (Cell Fix, BDIS, San Jose, USA). Samples were studied within 24 h on a flow cytometer platform (FacSort, BDIS, San Jose, USA). Data were analyzed using Cell Quest software (BDIS). Monocytes and neutrophils were gated according to CD45 *vs* side-scatter characteristics and CD33 or CD14 expression

intensity. CD33 and CD14 bright cells were assumed as monocytes by controlling their appearance on CD45-SSC, while CD33dim and CD14dim cells were evaluated as neutrophils. Isotypic control tubes and lymphocyte gates were used to set a marker for calculating TF-expressing cell percentage.

Statistics

The distribution of the laboratory data is expressed as mean ± standard deviation (s.d.). Student's *t*-test was used to assess the significance of differences between independent samples, and paired *t*-test was used for dependent samples. The relation of variables was tested using Pearson's correlation coefficient. A level of $P<0.05$ was accepted as statistically significant. The analysis was performed with SPSS 10.0 software.

Results

Baseline laboratory parameters (day 0) of all subjects were found to be within normal limits (Tables 1 and 2). Significant increases in WBC, neutrophil and monocyte counts were observed in all donors administered rhG-CSF as expected (day 5 *vs* day 0, $P<0.001$). There were no changes in red blood cell (RBC) or platelet counts at day 5 *vs* day 0 (Table 1). During rhG-CSF administration, collection of PBSC and follow-up period, we did not observe any thromboembolic event.

Table 1 Complete blood count and coagulation parameters before G-SCF (day 0) and after G-CSF (day 5) ($n=18$)

Variables	*Day 0 (mean ± s.d.)*	*Day 5 (mean ± s.d.)*	*P*
	Complete blood count		
Leukocyte (10^9/l)	7.3 ± 1.0	47.4 ± 13.8	<0.001*
Neutrophil (10^9/l)	4.27 ± 0.9	40.5 ± 13.2	<0.001*
Monocyte (10^9/l)	0.4 ± 0.1	2.1 ± 1.7	<0.001*
Red blood cell (10^{12}/l)	4.9 ± 0.5	4.7 ± 0.5	0.35
Platelet (10^9/l)	273 ± 86	271 ± 56	0.94
	Coagulation		
PT (s)	11.5 ± 0.7	12.0 ± 0.6	0.004*
PTT (s)	31.5 ± 3.7	31.4 ± 8.1	0.96
TT (s)	12 ± 1.0	14 ± 2.4	0.06
Fibrinogen (mg/dl)	339 ± 90.1	377 ± 72.2	0.17
D-dimer (ng/ml)	125 ± 92.1	188 ± 88.0	0.04*

*$P<0.05$.

Table 2 Plasma factor activities and natural anticoagulants of healthy donors before (day 0) and after G-CSF (day 5) ($n=18$)

Variables	*Day 0 (mean ± s.d.)*	*Day 5 (mean ± s.d.)*	*P*
Factor II (%)	108 ± 14.1	97 ± 16.0	0.03*
Factor VII (%)	126 ± 32.7	114 ± 63.7	0.49
Factor VIII (%)	131 ± 47.6	198 ± 63.7	0.001*
Factor IX (%)	136 ± 44.8	157 ± 75.0	0.32
Factor X (%)	118 ± 20.8	103 ± 15.5	0.02*
vWF:Ri CoF (%)	70 ± 24.0	90 ± 20.4	0.01*
ATIII (%)	108 ± 20.5	99 ± 19.4	0.17
Protein C (%)	106 ± 28.4	95 ± 8.6	0.09
Protein S (%)	100 ± 30.4	92 ± 28.2	0.42
APC-R	2.1 ± 0.3	2.0 ± 0.3	0.68

*$P<0.05$; APCR: activated protein C resistance.

Hemostatic parameters

As shown in Table 1, PT estimations were significantly prolonged from a mean value of 11.5 ± 0.7 to 12.0 ± 0.6 s after rhG-CSF ($P<0.01$). We observed a significant rise in D-dimer levels, with a slightly prolonged TT at day 5, but no relevant change in PTT and fibrinogen levels.

As shown in Table 2, a significant rise was observed in the mean activity of vWF:Ri CoF and FVIII, from 70 ± 24 to 90 ± 20%, but within normal ranges ($P=0.01$), and from 131 ± 47 to 198 ± 63% ($P=0.001$), respectively. However, the mean activity of FII and FX decreased significantly after rhG-CSF, from 108 ± 14 to 97 ± 16% ($P=0.03$) and from 118 ± 20 to 103 ± 15% ($P=0.02$), respectively. The mean activity of natural anticoagulants such as protein C, protein S and ATIII decreased after rhG-CSF to non-significant levels; there was no relevant change in the ratio of APC-R after rhG-CSF.

Plasma TF:Ag

There was a significant increase in mean plasma TF:Ag level from 34.3 ± 52 (0–185) to 100.4 ± 90 pg/ml (0–287 pg/ml) ($P=0.01$) (Figure 1). The increase in mean TF:Ag level was significantly higher in males (from 37.2 ± 58 to 122.8 ± 105 pg/ml, $P=0.04$) than in females (from 30.4 ± 47 to 70.9 ± 72 pg/ml, $P=0.20$) (Table 3), revealing an influence of the gender on TF activity.

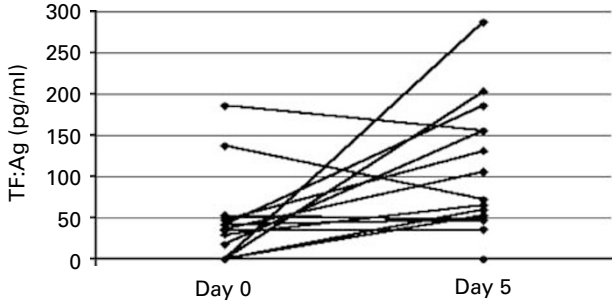


Figure 1 TF:Ag levels.

Table 3 Plasma TF:Ag and TF:PCA levels before G-CSF (day 0) and after G-CSF (day 5)

Variables	*Day 0 (mean ± s.d.)*	*Day 5 (mean ± s.d.)*	*P*
TF:Ag (pg/ml) ($n=18$)	34.3 ± 52	100.4 ± 92	0.01*
Female (pg/ml) ($n=8$)	30.4 ± 47	70.9 ± 72	0.20
Male (pg/ml) ($n=10$)	37.2 ± 58	122.8 ± 105	0.04*
TF: PCA (pg/ml) ($n=18$)	9.1 ± 20	48.9 ± 81	0.06
Female (pg/ml) ($n=8$)	5.2 ± 7	33.9 ± 71	0.27
Male (pg/ml) ($n=10$)	12.5 ± 27	62.3 ± 91	0.14

*$P<0.05$.

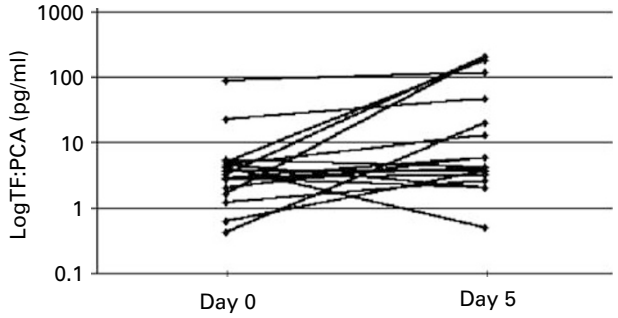


Figure 2 TF:PCA levels.

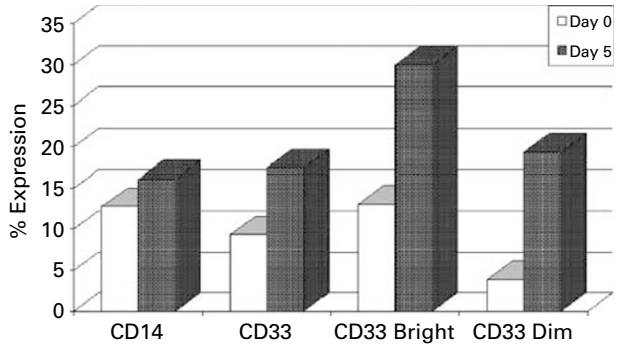


Figure 3 Comparison of mean TF:Ag expression.

Plasma TF:PCA

Plasma TF:PCA level increased from 9.1 ± 20 pg/ml (0.42–87 pg/ml) to 48.9 ± 81 pg/ml (0.50–210 pg/ml) after rhG-CSF, with a tendency to be near significant ($P = 0.06$) (Figure 2). Although this increase was more pronounced in males, there was no significant change in both genders (Table 3). No correlation between TF:Ag and TF:PCA could be detected either before or after rhG-CSF administration.

Cell surface TF antigen cytofluorimetric analysis

There was no change in TF:Ag expression on $CD14^+$ (monocytic) cells at day 5 *vs* day 0 (16.1 ± 23 *vs* $12.8 \pm 18\%$, $P = 0.37$). However, a significant increase in the level of TF:Ag expression on $CD33^+$ cells after rhG-CSF (from 9.3 ± 13 to $15.6 \pm 18\%$, $P = 0.04$) was observed. This increase was detected on both CD33bright cells and CD33dim cells, from 13.2 ± 22 to $30.1 \pm 32\%$ ($P = 0.04$) and from 3.9 ± 8 to $19.3 \pm 25\%$ ($P = 0.01$), respectively (Figure 3).

Discussion

In order to provide further insight into the contribution of rhG-CSF to hemostasis and thrombosis, we evaluated coagulation parameters of 18 healthy HSC donors. The mean activities of FII and FX were reduced by a ratio of 11–13% following rhG-CSF. Mean FVII activity also decreased slightly, resulting in prolonged PT. However, there was no change in mean FIX activity or mean PTT on day 5. This indicates that the coagulation factors in the extrinsic pathway were consumed rather than vitamin K-dependent factors. If rhG-CSF had affected vitamin K-dependent factors, we would have also observed a decrease in FIX activity and prolonged PTT. To date, there have been insufficient data about the effect of rhG-CSF on plasma coagulation factors. Following intravenous rhG-CSF administration to healthy donors, platelet aggregation response to adenosine diphosphate (ADP) and collagen increased, while D-dimer levels remained unchanged.[12] In our study, we found that the level of D-dimer, an indicator of an activated coagulation system, increased significantly from 125 to 188 ng/ml ($P < 0.05$), although values were within the reference range. Additionally, TT was prolonged at a nonsignificant level ($P = 0.06$), possibly as a result of increased fibrinogen turnover. A similar increase in D-dimer levels was also reported, although within the normal control range, at the first day of apheresis, using a higher dose of rhG-CSF (15 μg/kg/day).[13] Our study presents the first line of evidence that the coagulation cascade may be triggered following rhG-CSF administration by demonstrating a decrease in coagulation factor activity of extrinsic pathway. Although the prolongation of PT and elevation of D-dimer levels were found statistically significant in our study, all the values after rhG-CSF were within normal ranges.

rhG-CSF exerts an important effect on endothelial cells by inducing their migration and proliferation.[14] vWF and thrombomodulin (TM) are not only released from endothelial cells, but are also accepted as surrogate markers of stimulation and toxicity of the endothelium.[15] There have been several studies reporting that rhG-CSF increases the levels of vWF antigen, vWF activity and soluble TM.[11,16] A significant elevation of plasma vWF:Ri CoF and FVIII activity after rhG-CSF was found, although within normal ranges. The mechanism by which rhG-CSF exerts its effects on the endothelium has not been fully explained. Upon activation, neutrophils release reactive oxygen species and intracellular protease that perform several activities on endothelial cells and platelets and may modify the hemostatic balance toward a prothrombotic state,[11,17] to which a series of leukocyte-mediated events may have contributed, in addition to direct toxicity. As we did not investigate the relationship between levels of neutrophil-derived proteases and activation markers, we were not able to demonstrate the role of leukocyte-mediated endothelial toxicity.

The most common factor causing prothrombotic tendency is hereditary APC-R due to FV-Leiden mutation.[18] rhG-CSF did not result in APC-R phenotype in our study. Cumulative data about the effect of rhG-CSF administration on natural anticoagulants are still insufficient. It has been reported that the use of rhG-CSF after HSC transplantation had no negative impact on natural anticoagulants.[19] However, one study observing the effect of rhG-CSF on patients undergoing allogeneic stem cell transplantation showed a significant decrease in ATIII levels at the fifth day *vs* the transplant day.[20] Another study

of cancer patients showed that administration of rhG-CSF at different dosages (1, 3, 10, 30 and 60 μg/kg/day, i.v.) for 14 days did not affect ATIII levels.[21] After rhG-CSF, we observed a reduction of natural anticoagulants, remarkably for protein C levels, which were statistically not significant ($P = 0.09$). In contrast, a study using higher doses of rhG-CSF (12.5 μg/kg/day) on 25 healthy donors showed a significant increase in protein C and protein S levels and a slight decrease in ATIII levels.[22] Moreover, leukocyte-derived proteases such as elastase, which is vulnerable to rhG-CSF, have been known to suppress protein C, protein S and ATIII activities via proteolysis.[11] The increase in activated neutrophils as a result of rhG-CSF might have contributed to a reduction in the activities of natural anticoagulants.

At present, our knowledge about the contribution of growth factors to TF and/or TF-dependent pathway of coagulation is limited. Our cumulative data have a wide variation range, which can be explained by the so-called 'high and low responder phenomenon' related to differing response of monocytes to LPS stimulation.[23] We observed increases in the mean levels of both TF:Ag and TF:PCA in plasma after rhG-CSF ($P = 0.01$ and 0.06, respectively). However, there was no correlation between TF:Ag and TF:PCA at either day 0 or day 5. One of the possible reasons for this conflict may be the incomplete activation of TF:Ag. It has been suggested that with respect to procoagulant activity, the majority of cell surface TF activity is normally 'encrypted', in which state it is capable of binding antibody and FVII/VIIa, but not fully expressing antigenic activity. To become fully activated, it must be 'de-encrypted' by processes involving plasma membrane phospholipids and monomerization of TF.[24] In previous reports, rhG-CSF was shown to increase monocyte procoagulant activity.[11,13] No influence of age and gender on the plasma level of TF:Ag has been demonstrated in normal individuals.[25] In our study, the elevation of TF:Ag after rhG-CSF tended to be more pronounced in males (from 37 to 123 pg/ml, $P = 0.04$) than females (from 30 to 71 pg/ml, $P = 0.20$) (Table 3). The definitive role of TF in the development of atherosclerosis and an increased incidence in men must be evaluated within this context.

In normal individuals, TF:PCA is very low.[26] 'What is the source of TF:Ag and TF:PCA after rhG-CSF?' is a question to be answered. While there is debate over the contribution of neutrophils to TF:PCA[27,28], the main sources of TF in the circulation are considered to be monocytes and macrophages.[9,10] It has been put forward that circulating endothelial cells might have been contributing to TF:PCA during the aplasia period of stem cell transplantation because of the absence of mature myelomonocytes.[29] However, it was reported that a considerable amount of remaining TF:PCA originating neither from myelomonocytes nor circulating endothelial cells was detected during the peritransplant period.[30] The rise of endogenous rhG-CSF levels during the aplasia period of stem cell transplantation has been previously shown, but the effect of endogenous rhG-CSF levels on TF:PCA remains to be elucidated.[31] We observed a significant increase in cytofluorimetric surface TF expression after G-CSF on both $CD33^+$ dim (mature myeloid cells) and bright cells (monocytes and myeloid progenitor cells) without a major change in TF:Ag on $CD14^+$ cells (monocytes). The observation led us to suspect a possible contribution of neutrophils to TF:PCA beyond monocytes. While an increase of TF expression has been shown on rhG-CSF-treated neutrophils *in vitro*,[32] a clear correlation between the number of monocytes and increased TF expression has been demonstrated *in vivo*.[29,14] The acquisition of TF:Ag by neutrophils may have contributed to the increased TF levels in our study, and monocytes are likely to have played a role in this proposed mechanism; however, we were not able to clarify the contribution of a specific lineage to TF expression.

The prothrombotic state rarely encountered with the administration of rhG-CSF to both healthy donors and patients still needs to be clarified. Our study has provided evidence demonstrating that rhG-CSF increases the consumption of extrinsic pathway factors by stimulating coagulation by TF and the production of D-dimer, enhances vWF and FVIII levels by stimulating endothelial cells and reduces the activity of natural anticoagulants. Our study capturing a limited sample size cannot demonstrate a sole responsibility of rhG-CSF administration for peripheral blood stem cell collection at initiation of thrombotic events. Therefore, we propose that before administration of rhG-CSF to both donors and patients with known risk factors, a survey should be undertaken for evidence of inherited thrombophilia, including FV Leiden mutation, which has a quite frequent incidence of 3–10% in the population.

According to the results of our study on a limited number of PBSC donors, in individuals with underlying risk factors (hereditary and/or acquired thrombophilia), the responsible physician should consider the adverse effect of rhG-CSF on hemostasis by a TF-dependent pathway.

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