

# Role of Microparticles in the Hemostatic Dysfunction in Acute Promyelocytic Leukemia

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## ABSTRACT

Serious bleeding and thrombotic complications are frequent in acute promyelocytic leukemia (APL) and are major causes of morbidity and mortality. Microparticles (MP) have been used to study the risk and pathogenesis of thrombosis in many malignant disorders. To date, from published articles, this approach had not been applied to APL. In this article, the hemostatic dysfunction in this disorder is briefly reviewed. A study design to address this problem using MP is described. MP bearing tissue factor, profibrinolytic factors (tissue plasminogen activator and annexin A2), and the antifibrinolytic factor plasminogen activator inhibitor type 1 were measured using flow cytometry. The cellular origin of the MP was identified by specific cell surface markers. Comparison of the various populations of MP was made between samples collected at the time of diagnosis with those collected at molecular remission. Preliminary data suggest that this approach is feasible.

**KEYWORDS:** Acute promyelocytic leukemia, bleeding, thrombosis, microparticles, tissue factor; annexin A2

Despite the remarkable improvement in survival in patients with acute promyelocytic leukemia (APL) in the past 20 years,<sup>1,2</sup> a significant early mortality remains due mainly to bleeding and thrombotic complications. These complications are caused by a profound perturbation of the hemostatic balance. Recent findings indicate that the major factors contributing to the hemostatic dysfunction are increased expression of tissue factor (TF), cancer procoagulant, annexin A2, and tissue plasminogen activator (tPA) in the APL cells.<sup>3-6</sup> At the time of diagnosis of the disease, both the procoagulant and fibrinolytic factors are increased. Following treatment with a combination regimen of all-trans retinoic acid (ATRA) with anthracycline or of arsenic trioxide (ATO), most patients achieve a molecular remission,

along with reversal of all the hemostatic abnormalities. However, bleeding and thrombosis remain the major causes of early mortality. A new approach to investigate the pathogenesis of the coagulopathy is the study of microparticles (MP) in the plasma of APL patients. MP are cell fragments derived from a variety of cells. Their role in thromboembolic complications has been studied in many diseases. TF-bearing MP have been shown to contribute to the increased thrombotic risks in many forms of cancer. However, this approach has not been explored in APL. One reason may be the complexity of the components of MP involved in the thrombogenesis of this disorder. In this article, we briefly review the pathogenesis of the thrombotic and bleeding complications in APL. We then describe a proposed study

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designed to examine MP carrying TF as well as several fibrinolytic and antifibrinolytic factors in APL.

## ACUTE PROMYELOCYTIC LEUKEMIA

APL is a distinct subtype of acute myelogenous leukemia (AML),<sup>7</sup> formerly classified as (M3) by the French-American-British classification. It has molecular, morphologic, and clinical characteristics that distinguish it from other forms of AML. Molecularly, there is a balanced reciprocal translocation of chromosomes 15 and 17  $t(15;17)(q22;q12)$ , resulting in the fusion of the gene for retinoic acid receptor (RAR $\alpha$ ) on chromosome 17q12 with the gene for promyelocytic leukemia (PML) on chromosome 15q22, forming a PML/RAR $\alpha$  fusion gene in >95% patients with morphologically defined APL.<sup>8,9</sup> A key characteristic of APL is the blockage of granulocytic differentiation at the stage of promyelocytes, which is reversed by treatment with ATRA and/or ATO, making APL the paradigm of differentiation therapy.<sup>10</sup> Both drugs induce degradation of the PML/RAR $\alpha$  oncoprotein but through different mechanisms involving distinct residues of the PML (ATO) and RAR $\alpha$  (ATRA and ATO).<sup>10-12</sup> ATO is primarily an inducer of apoptosis *in vitro*, but at low concentrations and *in vivo* it also induces differentiation as does ATRA. In fact, both drugs act in synergism.<sup>13,14</sup> In rare cases of APL with  $t(11;17)$ , the RAR $\alpha$  gene is fused to PLZF and forms the PLZF-RAR $\alpha$  fusion protein.<sup>15,16</sup> This form of APL also shares similar clinical features with the classical variety of APL associated with  $t(15;17)/PML/RAR\alpha$ , but it is intrinsically resistant to ATRA but not to ATO.

Analysis of APL genesis using transgenic models revealed that the expression of the fusion protein is required to trigger the initiation of the disease, but the other mechanisms necessary for APL to fully develop are

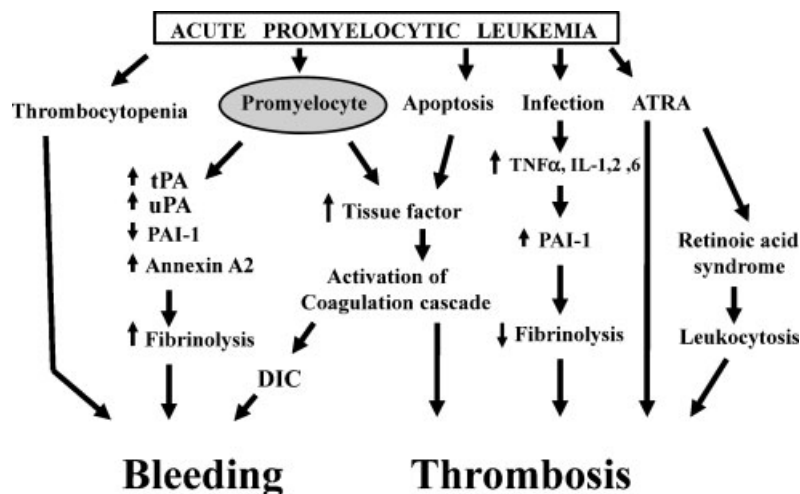
not entirely understood.<sup>17</sup> Additional gene deregulations or mutations can induce a block in myeloid differentiation or enhance APL development.<sup>18,19</sup> ATRA has been shown to reverse the expression of some of these factors. In addition, studies using oligonucleotide arrays indicate that genes associated with differentiation and with other cellular functions such as cell death and metabolism are modulated by ATRA.<sup>20</sup>

## Clinical Features

The age incidence shows a mean age of 30 to 38 years, rare <10 years of age, and declining after age 60.<sup>21</sup> The leukemic cells are characteristically arrested at the promyelocytic stage of myeloid differentiation. Morphologically, the cells have a reniform nucleus, prominent azurophilic granules, and many Auer rods. When analyzed by flow cytometry, the leukemic promyelocytes are usually negative for CD34, CD11b, CD14, HLA-DR, and positive for CD33. The microgranular variant occurs in 20% of cases and presents with leukocytosis instead of the leukopenia typical in the classic form.<sup>22,23</sup> Patients with thrombocytopenia and a high leukemic cell count have a worse prognosis.<sup>24</sup>

With a combination regimen of a differentiating agent (ATRA or ATO) and chemotherapy, complete remission rates can be achieved in >90% of patients.<sup>1</sup> However, this new therapeutic approach does not appear to decrease the overall early mortality rate. The cause of early deaths is primarily due to the high rate of bleeding complications.<sup>25-27</sup> Thus investigation of the pathogenesis of the hemostatic dysfunction in this disease becomes a matter of high priority.

In APL, there is increased risk of both bleeding and thrombosis, with the various contributory factors shown in Fig. 1. At presentation, APL patients fre-



**Figure 1** The hemostatic dysfunction in acute promyelocytic leukemia. DIC, disseminated intravascular coagulation; IL, interleukin; PAI-1, plasminogen activator inhibitor; TNF, tumor necrosis factor; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

quently show bleeding manifestations, and almost all patients have varying degrees of laboratory abnormalities in their coagulation parameters. The overall picture is one of disseminated intravascular coagulation (DIC). Of the bleeding complications, 65 to 80% are intracranial hemorrhages, usually fatal, followed by gastrointestinal bleeding and pulmonary intra-alveolar hemorrhage.<sup>28,29</sup> Paradoxically, thrombosis is also a major complication, creating a double hazard in this disorder.<sup>30-34</sup> Thrombosis occurred in 12% of cases in one series,<sup>35</sup> and in 15 to 25% patients in a postmortem study.<sup>36</sup> APL with the molecular features of the bcr3 isoform, CD2, CD15, FLT3-ITD, or is associated with a higher incidence of thrombotic complications.<sup>34</sup> Portal vein thrombosis is also seen in the microgranular variant of APL<sup>21</sup> expressing CD2.<sup>37</sup> Thrombosis can also be seen complicating the "retinoic acid syndrome" during differentiating therapy.<sup>38</sup>

### Laboratory Findings

The prothrombin time, partial thromboplastin time, and thrombin time are prolonged. Levels of fibrinopeptide A, prothrombin fragment 1 + 2, fibrin degradation products, and D-dimer are increased. The fibrinogen level and the platelet count are decreased.<sup>39-41</sup> The changes are reversed following differentiation therapy with ATRA in the first 4 to 7 days.<sup>39,41</sup> The fibrinolytic system is also perturbed with increased tPA and urokinase plasminogen activator (uPA), decreased plasminogen activator inhibitor (PAI)-1, and decreased  $\alpha$ 2-antiplasmin.<sup>41-45</sup>

### Pathogenesis of the Hemostatic Dysfunction

Alteration of the hemostatic balance in APL is usually severe and caused by multiple factors, including varying degrees of thrombocytopenia, increases in procoagulant activities of the leukemic promyelocytes, and alterations of the fibrinolytic system (Fig. 1).

#### INCREASED PROCOAGULANTS

The leukemic promyelocytes obtained from APL patients and the human promyelocytic leukemia cell line NB4 have been found to express high levels of TF<sup>46</sup> and of a cancer procoagulant (CP).<sup>46</sup> TF is the physiological initiator of coagulation activation cascade in both healthy individuals and in malignant tissues.<sup>47</sup> The procoagulant activity of TF on the cell surface is largely dormant (TF encryption) until alterations of the plasma membrane occur. Thus disrupted cells generate more procoagulant activity than intact cells.<sup>48</sup> One explanation is that TF encryption is a result of sequestration of phosphatidylserine, which acts as a cofactor for TF procoagulant activity. TF expression is increased in APL promyelocytes. The procoagulant activity of lysates

from freshly isolated APL cells is mainly attributed to TF, whereas CP only exerts a minimal effect. APL cells may also induce TF procoagulant activity of endothelial cells through their secretion of interleukin (IL)-1 $\beta$ . The addition of ATRA to cultures of NB4 cells in 1  $\mu$ M concentration, comparable with the level in a treated patient, restores the TF to normal (unpublished data).

During the course of APL, the procoagulant activity shows periods of exacerbation. At the onset, there is progressive proliferation along with apoptosis of the promyelocytes. The apoptosis is then further enhanced by treatment with ATO or chemotherapy, and to a much lesser extent with ATRA. As the leukemic cells undergoing apoptosis, or necrosis, exteriorization of the phospholipids, especially phosphatidylserine, on the cell surface leads to activation of the encrypted TF. Thus apoptotic cells are more thrombogenic.<sup>49</sup> This may be an important factor in the clinical observations in the pre-ATRA days that the bleeding manifestations were exacerbated during chemotherapy.

#### CHANGE IN THE FIBRINOLYTIC SYSTEM

The normal balance between profibrinolytic and antifibrinolytic factors is altered in APL. Several events may contribute to the increased fibrinolysis. A secondary fibrinolysis may occur as a response to DIC at the onset of the disease. In APL, the promyelocytes themselves overexpress both tPA and uPA. In addition, annexin A2, a coreceptor for plasminogen and for tPA, was also found to be highly expressed in APL promyelocyte and in NB4 cells.<sup>50</sup> The expression of these factors is down-regulated after ATRA treatment. Thus overall there is an increase in the fibrinolytic activity at presentation. It is uncertain how much this can counteract the procoagulant activity in the clinical setting. However, the increased fibrinolytic activity is a major factor in the bleeding complications. The preponderance of intracranial bleeding in APL may be explained by a higher expression of annexin A2 in the microvascular endothelial cells derived from the brain when compared with that in endothelial cells in other parts of the body.<sup>51</sup> Such high expression of this receptor of plasminogen and of tPA might attract more fibrinolytic components to the brain. In the presence of annexin A2, plasmin generation is increased by as much as 60-fold.<sup>50</sup>

Because annexin A2 is increased in the leukemic promyelocytes, the degree of fibrinolytic activity is proportional to the leukemic burden. This is supported by the observation that severe bleeding complications are correlated with the white cell count at presentation.<sup>35</sup>

In contrast, the antifibrinolytic factor PAI-1 is upregulated in APL.<sup>41</sup> PAI-1 is a potent inhibitor of tPA and uPA. The increase in PAI-1 is variable and depends on several factors. Inflammatory cytokines, especially tumor necrosis factor  $\alpha$  and IL-6, upregulate the expression of PAI-1.<sup>52</sup> Thus, in the presence of

infection or sepsis in APL, the PAI-1 level is increased. Conversely, leukocyte elastase and other proteases from the leukemic promyelocytes degrade PAI-1 into an inactive form,<sup>43</sup> leading to decreased PAI-1 activity in the plasma. Hence contradictory findings have been reported in the literature when the data are not analyzed according to the status of infection or the severity of the DIC. If the level is abnormal at presentation of the APL, it will return to normal with differentiation therapy.<sup>3,41</sup>

Recent evidence shows that tPA and PAI-1 expression in endothelial cells is regulated by post-translational histone modifications.<sup>52</sup> Also, retinoids (ATRA) can induce tPA expression in astrocytes.<sup>53</sup> Whether these findings apply to APL leukemic cells is an intriguing question because they may be another factor in the altered fibrinolytic activity in APL.

## MICROPARTICLES

Microparticles (MP) are cell-derived fragments 0.1 to 1.0  $\mu\text{m}$  in diameter. They are present in the circulating blood and derived from multiple sources such as platelets, red blood cells (RBC), white blood cells (WBC) (neutrophils, monocytes), and endothelial cells in healthy subjects and from atherosclerotic plaques and from tumor cells in cancer patients. Studies of several cellular proteins have been performed on MP for the investigation of the hemostatic dysfunction in cancer, cardiovascular diseases, and autoimmune disorders. These include cytokines and components of the coagulation and fibrinolytic systems. The risk of thromboembolic events has been correlated to TF-bearing MP in patients with various forms of cancer, including lung, colorectal, breast, and pancreas. In some studies these MP were derived from circulating platelets, whereas in others, they originated from tumor cells. Most studies centered on measurement of TF antigen by flow cytometry. Others used MP isolated by centrifugation for the study of TF activity and of thrombin generation.

### How the Study of Microparticles Can Be Used in Acute Promyelocytic Leukemia

Before the introduction of techniques for studying MP, investigations of hemostatic abnormalities were performed with plasma. The plasma level of TF has often been used as an indication of the thrombotic state of the patient, with little information on whether the TF originates from platelets, endothelial cells, tumor cells, or even atherosclerotic plaques. However, information on the cellular origin of TF-bearing MP can be obtained by using specific cellular surface markers.

In healthy subjects, MP in the plasma are mostly derived from platelets<sup>54,55</sup> (around 60 to 80% of all the MP), with  $\sim 10\%$  derived from erythrocytes and leuko-

cytes, respectively. These individual populations vary widely, depending on whether or not there is activation of any of these cellular elements. Among the MP in the circulating plasma in healthy subjects, those bearing TF account for only  $\sim 12\%$ .<sup>55</sup> These MP are derived mostly from platelets and monocytes. In different diseases, profound changes have been observed. For example, in cardiovascular diseases, TF-bearing MP originate from endothelial cells. In APL, the leukemic promyelocytes express a high level of TF. Likewise, those MP derived from the leukemic cells also carry TF. Thus the populations of TF-bearing MP in APL plasma are correspondingly increased. In patients at the time of diagnosis, circulating MP are mostly from the APL promyelocytes, which can be identified by the surface marker CD33. At the same time, due to the presence of thrombocytopenia, the population of platelet-derived MP (identified by CD41) is reduced. With response to treatment, the population of promyelocytes decreases and the platelet counts return to normal. The distribution of the MP population changes correspondingly. The predominant MP now become those derived from platelets. In other words, if the TF-bearing MP are measured, they will be mostly derived from the leukemic promyelocytes at the time of diagnosis, and after remission, the TF-bearing MP will be switched back to those derived from platelets and from monocytes.

Other relevant proteins on the MP are tPA, PAI-1, and annexin A2 because they have all been implicated in the thrombogenesis and bleeding in APL. These proteins are derived from many cell types, including endothelial cells and monocytes. The latter two cell types are identified by their respective surface markers, CD31 and CD14.

The complexity of these variables was demonstrated in an ongoing study of APL patients treated in a multicenter protocol. They were accrued by the International Consortium on Acute Promyelocytic Leukemia (IC-APL) under the auspices of the American Society of Hematology. The study protocol and the methodology of assaying the MP are as follows.

## PROTOCOL

Adult patients  $< 70$  years of age with the suspected diagnosis of APL by morphology were immediately started on ATRA while bone marrow samples were shipped to a national central laboratory in which genetic verification of the diagnosis was performed. Induction treatment consisted of ATRA 45  $\text{mg}/\text{m}^2$  per day until hematologic complete response and chemotherapy with daunorubicin (DNR) 60  $\text{mg}/\text{m}^2$  per day for 4 days. The patients were stratified according to the PETHEMA/GIMEMA risk categories.<sup>56</sup> Those in the low-risk group received as consolidation DNR 25  $\text{mg}/\text{m}^2$  per day for 4 days and ATRA 45  $\text{mg}/\text{m}^2$  per day for 15 days in the first course, Mitoxantrone (MTZ) 10  $\text{mg}/\text{m}^2$  per day for



3 days and ATRA 45 mg/m<sup>2</sup> per day for 15 days in the second course, and DNR 60 mg/m<sup>2</sup> per day for 1 day and 45 mg/m<sup>2</sup> per day for 15 days in the third course. Patients allocated in the intermediate risk group received the same consolidation except that the dose of DNR in the first course was of 35 mg/m<sup>2</sup> per day, and in the third course DNR 60 mg/m<sup>2</sup> per day was administered for 2 days. Patients in the high-risk group received the same treatment described for the low-risk group except that Ara-C 1000 mg/m<sup>2</sup> per day for 4 days was added in the first course and Ara-C 150 mg/m<sup>2</sup> for 8 hours for 4 days was added in the third course of consolidation. All patients received maintenance therapy, which consisted of 6-mercaptopurine 50 mg/m<sup>2</sup> per day, methotrexate 15 mg/m<sup>2</sup> per week, and intermittent ATRA 45 mg/m<sup>2</sup> per day for 15 days every 3 months. Maintenance therapy was continued for 2 years. Supportive care aimed at maintaining platelet counts > 30,000/μL and fibrinogen levels > 150 mg/dL.

## METHODS

Two blood samples were collected, the first at the time of diagnosis and the second at the time of molecular remission. This was at the end of the induction therapy in all the patients studied. Blood was collected into citrated Vacutainer tubes. Platelet-poor plasma (PPP) was prepared by centrifugation at 1500 g for 15 minutes and stored at -70°C. MP were obtained from PPP by centrifugation at 13,000 g for 10 minutes. MP derived from 1 mL of PPP were resuspended in 100 μL of binding buffer (50 mM Tris, 80 mM NaCl, 0.2% BSA). TF antigen was identified by fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against human tissue factor (American Diagnostica, Greenwich, CT). Annexin A2 antigen was identified by monoclonal antibody against human annexin A2 labeled with Alexa Fluor 647 (both from Zenon Invitrogen, Carlsbad, CA). CD33 antigen was identified by anti-CD monoclonal antibodies labeled with PE-Cy7 (both from Becton Dickinson, San Jose, CA). The sample was incubated in the dark at room temperature for 30 minutes before flow cytometric analysis. Following manufacturer's protocol, phycoerythrin (PE) annexin V antibody was added (BD-Pharmingen, San Jose, CA; PE apoptosis detection kit) for an additional 15 minutes after which the volume was brought up to 500 μL with binding buffer for analysis by flow cytometry. All samples were run concurrently with plasma from healthy donors.

Flow cytometric studies were conducted on three-laser Beckman Coulter Cyan running Summit Software. Due to better resolution between debris and MP in side scatter compared with forward scatter, electronic triggering was done on side scatter to eliminate debris. FSC and SSC channels were calibrated using various sizes of latex beads. The beads ranged in size from 0.1 μm to 1

μm. Events smaller than 0.3 μm were eliminated from analysis due to the inability to distinguish from background noise. Further enrichment of MP was done by first gating only the annexin V positive events. Concentration of MP was determined by adding AccuCount fluorescent particles (Spherotech, Lake Forest, IL) as an internal control. Sample acquisition was stopped after 2000 AccuCount beads were detected. With this information, the volume of each sample collected was calculated and concentration was therefore determined. Proper gates to determine positive MP were defined using isotype controls. The amounts of a specific type of MP are expressed as a percentage of the total number of events.

## STUDY DESIGN

This study was designed to determine whether differentiation treatment by ATRA affects the hemostatic dysfunction in APL, using MP taken from patients at the time of diagnosis and at molecular remission. Preliminary results (to be published) indicate the total number of TF-bearing MP in the plasma is greatly increased in APL samples up to 10-fold higher than in healthy subjects. Unexpectedly, the high value persists immediately following remission of the disease when there are no leukemic cells in the blood. Further analysis of these TF-bearing MP revealed that those derived from CD33 (promyelocytes) cells show a decrease in TF but are still far from being normalized. It is not presently known when normalcy can be achieved. The TF-bearing MP at remission are not limited to those derived from CD33 cells. They can originate from other cells such as the platelets, monocytes, and endothelial cells. With this study design, the proportion of TF-bearing MP from each of these cellular origins can be obtained. This will bring new information on what thrombogenic role each of these cells play.

When the profibrinolytic factors annexin A2 and tPA are measured, a similar pattern is observed. Annexin A2 levels are 10-fold higher than in healthy subjects, and again, these high levels persist after remission. The annexin A2 levels in the MP derived from APL promyelocytes also decrease in most patients in remission. The tPA levels are 15-fold higher than in healthy subjects and continue high after remission, as do the PAI-1 levels. The clinical implication of these preliminary findings, if confirmed, is that the risks for both thrombosis and bleeding persist even in remission. This may be explained by the fact that all the postremission samples were collected at the end of induction chemotherapy. Thus a longer follow-up is needed to see when the changes revert to normal.

There are other advantages to studying MP. They can be viewed as carriers of proteins and cytokines from their respective cellular origins to other parts of the body. They are known to be phagocytosed by macrophages in

the liver and spleen.<sup>56,57</sup> They can also bind to other MP. For example, leukocyte MP bearing TF and P-selectin glycoprotein ligand 1 bind to P-selectin on endothelial cells and to MP derived from endothelial cells as well.<sup>58</sup> Through such binding, proteins and other substances may be transferred. MP from endothelial progenitor cells have been observed to transfer mRNA to endothelial cells promoting angiogenesis.<sup>59,60</sup>

## CONCLUSION

Bleeding and thrombosis remain major causes of morbidity and mortality in APL. Our understanding of the pathogenesis of these complications is still incomplete. The use of MP technology may offer a new avenue for future studies by providing fresh information on the sources of the procoagulant and fibrinolytic factors in APL.

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