A Coagulation Factor Becomes Useful in the Study of Acute Leukemias: Studies with Blood Coagulation Factor XIII

Flóra Kiss,1 Ágnes Simon,1 László Csáthy,1 Zsuzsanna Hevessy,1 Éva Katona,2 Csongor Kiss,3 János Kappelmayer1*

Abstract
The intracellular form of the coagulation factor XIII has previously been identified by immunomorphological techniques using polyclonal antibodies. In these studies, only the A subunit (FXIII-A) was detectable in megakaryocytes/platelets and in monocytes/macrophages. We developed several novel monoclonal antibody clones directed to both subunits (FXIII-A and FXIII-B) and investigated their appearance in normal and leukemic cells. By using 3- and 4-color flow cytometry FXIII expression was investigated in normal peripheral blood and bone marrow samples and in acute myeloblastic (AML) and lymphoblastic (ALL) leukemia cases. Samples were studied by Western blotting and confocal laser scanning microscopy. With a previously published ELISA assay applying two monoclonal antibodies directed to different epitopes in FXIII-A, we were able to measure the intracytoplasmic content of FXIII-A in normal cells and leukemic blasts. FXIII-A was detectable in normal peripheral blood monocytes and in large quantities in platelets, but both cell types were negative for FXIII-B. There was no surface staining for FXIII-A, it only appeared intracellularly. In samples derived from patients with AML M4 and M5, FXIII-A sensitively identified blast cells. Although normal lymphocytes do not express FXIII-A, 40% of ALL cases showed significant FXIII-A expression as determined by flow cytometry. FXIII-A positivity of lymphoblasts was verified by Western blotting, ELISA, and confocal laser scanning microscopy cytometry. These data provide evidence that FXIII-A is a sufficiently sensitive marker in differentiating myeloblasts and monoblasts and is suitable for identifying leukemia-associated phenotypes in ALL.

Key terms
factor XIII; flow cytometry; acute leukemia phenotype

Hematological malignancies are exceptional in the sense that unlike solid tumors, right from the beginning they are spread all over the body since cancer cells appear in the blood. Acute leukemias represent 85% of all leukemias in children and 45% in adults. In childhood, ~80% of acute leukemias are lymphoblastic and 15–20% are myeloblastic (1), whereas this proportion is basically reversed in adults. Leukemic cells display phenotypes that are either present in normal precursors or is unique for the leukemic population also referred to as leukemia-associated immunophenotype (LAIP) or aberrant phenotype. Several publications described LAIP on blasts in acute leukemias (2–5). The major features are lineage infidelity and lineage promiscuity, where leukemic cells express antigens associated with a different cell line, e.g., CD13 and CD33 myeloid-associated antigens in precursor-B acute lymphoblastic leukemia (ALL) (6) or CD7 T-cell marker expression in neoplastic myeloblasts (7). In case of asynchronous differentiation, markers of different maturational stages are expressed simultaneously, like the coexpression of CD10 and CD20 in B-ALL (8) or CD117/CD15 coexpression in acute myeloid leukemia (AML) (9). Antigen...
overexpression is an aberrantly high expression of an antigen also present in normal cells and the most typical example is the overexpression of CD10 in precursor-B-ALL (10).

Since leukemic blasts appear in the circulation they evidently can interact with several plasma proteins including coagulation factors. Mostly, this interaction is an association of coagulation factors with the blast cell surface; however, in some cases the intracytoplasmic form of a clotting factor has also been found. The major events in the coagulation cascade and their cellular interactions are briefly summarized below.

**THE COAGULATION CASCADE**

The final step of the coagulation process is the formation of the crosslinked fibrin in thrombi and this is achieved by the amplification process of the clotting cascade symbolized by the waterfall mechanism described by Davie and Ratnoff (11) and Macfarlane (12). The major initiator of the coagulation cascade is the transmembrane protein tissue factor (TF). TF is normally expressed on several cell types of different organs such as adventitial fibroblasts and pericytes, astrocytes of the brain, epithelial cells of the renal glomeruli, cardiomyocytes, epithelial cells enveloping organs and body surfaces (13–15); however, none of these are normally in contact with blood. TF can be exposed to flowing blood in three ways (i) upon endothelial damage, (ii) pathophysiological stimuli can induce peripheral blood monocytes and endothelial cells to express TF, and (iii) certain leukemic (or tumor) cell types can appear in the peripheral blood that express TF (16,17). It is evident that all these cases would activate the downstream cascade of clotting factors. First TF will bind and activate factor VII (FVII) a serine protease on a phospholipid surface. This TF-FVII complex will activate another multiprotein complex the prothrombinase complex. This is comprised of two clotting factors: factor V a procofactor and factor X a serine protease. The TF-FVII complex can also activate factor IX (18). The prothrombinase complex—also on a phospholipid surface—in the presence of calcium will convert the zymogen prothrombin to thrombin and thus the central protease of the coagulation cascade will be formed (Fig. 1). Thrombin displays multiple effects on the clotting cascade that are mostly thrombotic, including platelet activation, however, some actions are antithrombotic as well depending on the interaction with specific receptors (19,20). Hemostatic processes are strictly controlled and the formed thrombin is inhibited by antithrombins, but if

---

**Figure 1.** The blood coagulation cascade showing the extrinsic and intrinsic pathways, fibrinolytic pathway. Activation and inhibition signals are differentially displayed, “do not enter” sign and dashed line stand for inhibition. HMWK, high molecular weight kininogen; TFPI, tissue factor pathway inhibitor; PI, phospholipid surface; tPA, tissue plasminogen activator.
generated in excess, thrombin splits fibrinogen to fibrin. Fibrin monomers are converted to fibrin polymers that are crosslinked by activated factor XIII (21), and thrombin also has a very important role in activating factor XIII. Fibrin in physiological states is lysed by the major fibrinolytic protease plasmin. Plasmin formation is also regulated by activators and inhibitors.

**ACUTE LEUKEMIAS AND THE COAGULATION CASCADE**

Cancer cells can interact with clotting processes both directly and indirectly. The direct form could be the expression of TF on the tumor cell surface and the formation of the cancer procoagulant that is a direct factor X activator. A high proportion of patients with acute leukemia have coagulation abnormalities at the time of presentation which contribute to both thrombotic and bleeding tendency (22–24). The pattern of routine coagulation test abnormalities in the majority of patients with acute promyelocytic leukemia (APL) at presentation is consistent with intravascular coagulation activation. Elevated values of thrombin–antithrombin complexes and fibrinopeptides are detectable indicating the presence of thrombin generation and the conversion of fibrinogen to fibrin. The development of intravascular coagulation in APL has been attributed to the presence of TF by the promyelocytic leukemia cells resulting in FVII activation. In acute leukemias, in addition to TF, a cysteine proteinase has been described that is detectable in acute myeloid leukemia blasts and is most abundant in APL. The cellular composition of APL blasts, however, enables these cells to interact with clotting processes in multiple ways. One such protease present in APL blasts is elastase that on the one hand can generate fibrin by cleaving the A-alpha chain of fibrinogen and on the other hand can contribute to a nonplasmin mediated fibrinolysis (23,24) (Fig. 2).

**FACTOR XIII EXPRESSION IN NORMAL CELLS**

The cellular appearance of FXIII has not been evident for quite some time. More than a decade after the discovery of the plasma transglutaminase, Buluk described the appearance of factor XIII in platelets and megakaryocytes (25). However, contrary to plasma, in cells only the A subunit of the enzyme (FXIII-A) appears and the inhibitory B subunits are undetectable. The cellular localization of FXIII-A got a significant boost when two groups independently reported the appearance of FXIII-A in peripheral blood monocytes in 1985 (26,27). This discovery helped in the clarification of the localization of FXIII-A in the liver that has previously been thought to be localized in fibroblasts (28). However, mostly by works of Adany et al., it has been described that tissue macrophages are source of FXIII-A (29,30), and fibroblasts are devoid of...
FXIII activity. FXIII-A expression was also found in osteoblasts of the bone (31). It is important to note that the sole detection of transglutaminase activity in cellular extracts does not directly imply the involvement of FXIII-A, since tissue transglutaminase (tTG) has been shown in red blood cells, hepatocytes, and vascular endothelial cells (32,33). This single chain intracellular calcium-dependent enzyme also cross-links fibrin(ogen) but unlike FXIII-A, tTG cross-links α-chains in preference to γ-chains (34,35). Although the exact physiological role of tTG is still uncertain, several functions of tTG have been proposed: (i) extracellular matrix modification (36), (ii) involvement in apoptosis (37,38), and (iii) action in endocytosis (39) and cell signaling (40). Thus, the immunological verification of FXIII-A either by morphological techniques or Western blotting is the method of choice to undoubtedly detect this protein. In addition, the role of intracellular FXIII-A has not been clarified and an embarrassing phenomenon is the potential role of FXIII-A in platelets. These cells carry half of the FXIII-A activity in the blood and contain 100–150 times more FXIII-A than plasma per volume (41). Previous studies with platelets described only the binding and not the release of FXIII. However, these results were contradictory whether FXIII is bound to the GPIIb/IIIa complex on the platelet surface (42–44). The detection of FXIII-A has been described in normal bone marrow precursors by morphological techniques and has been found more sensitive to detect premature bone marrow monocytes than CD14, a universally used monocyte/macrophage marker (45). It is important that when normal peripheral blood monocytes and platelets are investigated, FXIII-A is not detectable on the cell-surface but only intracellularly. Permeabilization of cells can be technically demanding particularly in cases of complex cell populations like bone marrow. Thus we first optimized the utilization of permeabilization techniques (46) and used the optimal conditions for FXIII-A labeling in subsequent studies.

**Factor XIII Expression in AML**

The expression of FXIII-A in tissue specimen has been investigated in multiple disorders (47,48). However, in these studies FXIII-A mostly did not appear in the malignant cells, but in macrophages and follicular dendritic reticulum cells (DRCs), sinusoidal and interfollicular histiocytic reticulum cells associated with the tumor. On the basis of the results of normal cells, it was presumable that malignant counterparts of megakaryocytes and monocytes also express FXIII-A. This has first been shown in 1992 by Invernizzi et al. by using immunomorphological techniques in case of acute monocytic leukemias (49). Today the generally accepted methodology of leuke-
mia phenotyping is multicolor flow-cytometry. We found that
in case acute monocytic and myelomonocytic leukemias
FXIII-A was more sensitive than CD14 in detecting leukemic
blasts (Fig. 3). In disorders where more mature monocytes are
found like chronic myelomonocytic leukemias, FXIII-A and
CD14 labelings were identical. This is also evident when cell
lines are analyzed since in the monoblastic MonoMac6 cells,
FXIII-A is constantly expressed intracellularly while CD14

Figure 5. Washing with EDTA-
containing PBS eliminated false-
positive reaction, due to platelet
adhesion to white blood cells,
leaving only AML M7 blasts (A, C)
positive for platelet marker CD41
(GpIIb), while FXIII-A expres-
sion was unchanged (B, D). The
red cell cluster represented the
CD45dim/CD41+/FXIII-A+ AML
M7 blasts. The blue cluster rep-
resented the lymphocytes with
bright CD45 expression,
whereas the green cluster
showed CD45+ neutrophils. A
moderate, false CD41 positivity
was observed on these white
blood cells due to platelet adhe-
sion (A, C).

Figure 6. Representative flow
cytometric scattergrams of
lymphoid cells from a FXIII-A
positive ALL (A) and normal
bone marrow sample (B). ALL
sample was labeled by FXIII-
A(FITC)-CD34(PE)-CD45(PerCP)
staining. FXIII-A positive blasts
coexpressed CD34 and FXIII-A
(see A/1, upper right quadrant).
appears only upon induction with vitamin D. The reaction was specific as in the myeloblastic PLB-985 cells, FXIII-A was always negative even upon maturation. It is very important to note that in case of FXIII-A the intensity of staining is enhanced in malignant cells unlike other cytoplasmic markers like myeloperoxidase (Fig. 4). When AML M7 cases were studied we found that FXIII-A labeling is more specific than the generally utilized platelet markers. Conventionally used platelet/megakaryocyte markers, such as CD41, CD42a/b, often display false-positivity in case of nonmegakaryocytic leukemias (50), or in case of extracorporeal circulation and reperfusion (51). Circulating microparticles, a very heterogeneous group of cell fragments, can also be the cause of false platelet marker positivity (52). In de novo M7 cases fairly high GPIIb and GPIX expressions, can also be the cause of false platelet marker positivity (53). In de novo acute leukemic patients we found FXIII-A and platelet glycoprotein markers match (Fig. 5). This means that FXIII-A depicts the true megakaryocyte fraction in these cases. Similarly, in normal samples, this washing procedure was useful to eliminate false platelet marker positivity.

**FACTOR XIII EXPRESSION IN ALL**

Normal lymphoid cells do not synthesize or contain either subunit of FXIII as has been shown previously (26,53). Surprisingly, however, in bone marrow samples derived from de novo acute leukemia patients we found FXIII-A positivity in 40% of ALL patients by 3-color flow cytometry (54). In these cases FXIII-A positivity was coexpressed with CD45dim/CD34+ labeling (Fig. 6) and B-cell markers such as CD19 and cytoplasmic CD79a. This finding was unexpected since normal peripheral blood lymphocytes as well as normal lymphoid precursors in the bone marrow are devoid of FXIII-A labeling. Thus, human peripheral blood taken from a healthy donor was analyzed by flow cytometry. The purity of the CD34+ cells was 98% after magnetic separation in the leukapheresis product. In normal B-lymphocyte precursors—CD34 and cyCD79a—, positive events were found in 3%. In these B-lineage committed cells no FXIII-A could be detected. In addition, FXIII-A was undetectable in lymphocytes derived from chronic lymphocytic leukemia (data not shown). To further verify the presence of FXIII-A in ALL blasts, from six samples that contained over 90% of lymphoblasts and no detectable monocytes at diagnosis, cell lysates were prepared. To avoid platelet contamination lymphoid cells were washed 3 times in PBS containing 20 mM EDTA. Two methods were used to identify FXIII-A in these cells, Western-blotting and ELISA. The exact cell count was determined before sonication in order to calculate the amount of FXIII-A content/cell. By Western-blotting it was found that under reducing conditions FXIII-A appeared as a 82 kD protein band in ALL blasts but not in mature B-cells derived from patients with CLL. The band obtained in case of ALL blasts, however, seemed much fainter than that obtained for platelets. It is known that platelets contain very large quantities of FXIII-A (41). Thus, we tried to compare the results to that of monocytes that were purified from buffy coats by positive selection using anti-CD14 magnetic beads and VarioMACS (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The ELISA technique used two monoclonal antibodies that detect different epitopes in the FXIII-A subunit and has been previously described to be suitable to assay FXIII-A in cell lysates (55). By ELISA we found 3.1 ± 1.2 fg/blast (range: 2.0–4.8 fg/blast) that is 10% of the quantity seen in monocytes. However, considering the significantly larger cell volume of monocytes this quantity is not negligible. Finally, we used confocal laser scanning microscopy to localize FXIII-A on cytoplasms and in cell suspensions. In both sample types FXIII-A appeared with a similar staining pattern to normal monocytes and nuclei were devoid of FXIII-A staining (Fig. 7). The FXIII-A-lymphoblasts were negative for the platelet markers GPIIb and GPIX.
**LITERATURE CITED**


