THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Expression of coagulation factor XIII in acute myeloid leukemias

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INTRODUCTION

The Structure of Factor XIII (FXIII), Description of Plasmic and Cell Factor XIII

Factor XIII (FXIII) of blood coagulation, a protransglutaminase, becomes activated by the proteolytic action of thrombin in the presence of calcium. Beside calcium the process is controlled by fibrin(ogen). The activated form of the enzyme (FXIIIa) is responsible for cross-linking of fibrin strands, thus stabilizes the clot in the final stage of the coagulation process.

Over the clotting process the circulating precursor of the proteolytic enzymes becomes activated by a cascade-like proteolysis. The strongest stimulus that starts the process is tissue factor (TF) on the cell surface. TF is expressed by various cell types, for instance, by fibroblasts and pericytes in the vessel wall or astrocytes of the brain, or the epithelial cells of kidney glomeruli, or myocardial cells. These cells are however, in touch with blood components only in case of endothelial damage. In circulating blood, the monocytes and the endothelial cells of vessels can express TF. In addition, circulating TF-expressing tumor cells may activate the clotting cascade. In the presence of phospholipids and Ca^{2+}, TF activates FVII, which is responsible for activating FV and FX that build up the prothrombinase complex. The TF-FVIIa complex also activates FIX. Under the influence of prothrombinase complex, prothrombin transforms into the active protease, thrombin. Thrombin activates not only platelets and FXIII, but it transforms fibrinogen to fibrin. Robbins et al described that calcium was not sufficient to make the coagulated fibrin insoluble in weak acids or bases. They postulated the existence of a further factor in the serum. The presence of the serum factor needed for the stabilization of the fibrin clot was first described by two Hungarian researchers Kálmán Laki and László Lóránd in 1948. They called this protein 'fibrin-stabilizing-factor'. Later, it was Loewy and his co-workers who purified it and described its enzymatic characteristics. After identifying the first deficiency case, it was acknowledged as a new factor of blood coagulation with the serial number 'XIII' in 1963.

The Expression Sites of Factor XIII Subunit 'A and B'

Two forms of the FXIII exist in humans. One of them circulates in the blood as a heterotetramer, the other one is present in cells as homodimer (A_2) consisting of two 'A' subunits. The heterotetramer form is composed of two potentially active 'A' (FXIII-A) and two carrier/protective 'B'(FXIII-B) subunits (A_2B_2). The 'B' subunit prevents the activation of the 'A' subunits. Liver cells synthesize the 'B' subunit, while megakaryocytes, platelets

and cells of the monocyte/macrophage system synthesize subunit ’A’. To a small extent liver cells can also synthesize. Among other types of cells the FXIII-A synthesizing capability of the chondrocytes has also been verified and it has also been detected in osteoblasts. Approximately 50% of the FXIII-B amount being in the blood circulates freely in the plasma, while the entire amount of FXIII-A can be found in the tetramer complex. The mean plasma concentration of FXIII complex is 21 mg/L. The conditions of the formation of FXIII-A_2B_2 complex has not been clarified yet.

Intracellular Localization Sites of the FXIII-A (Subcellular Distribution)

Platelets contain a huge amount of FXIII-A, 150-fold more per volume than plasma, while the FXIII-A concentration in monocytes is at least one order of magnitude less than that in platelets. It has been demonstrated that common bone marrow precursor cells of monocytes and megakaryocytes also express FXIII-A The function of the intracellular enzyme has not yet been elucidated, but many experimental results have shown that the megakaryocytes, monocytes and macrophages are not the only sites of FXIII-A synthesis, but they play an active role in pathophysiological processes of the cell.

FXIII-A displays a typical cytoplasmic distribution within macrophages. It can be observed around the cytoplasmic vacuoles and in the pseudopodia, however, it can not be found in phagocytic vacuoles. In the early stage of monocyte/macrophage maturation the nuclear accumulation of XIII-A was observed in these cells. A role in the phagocytosis and in the alternative activation of macrophages was also supposed. In platelets, FXIII is bound to HSP27, a heat-shock protein.

FXIII-A Appearance in Malignancies

Apart from cells of the megakaryocyte/platelet or monocyte/macrophage system, FXIII-A can not be detected in any other bone marrow or peripheral blood cells. Moreover, the presence of FXIII-A was examined in case of several other illnesses and malignant diseases. Fibrin depositions with FXIII were observed in lymph nodes of patients suffering from Hodgkin's lymphoma with abundant macrophage presence. This cell population contained FXIII-A and these cells were identified as tumor-associated macrophages. The appearance of FXIII-A in malignant fibrous histiocytoma (MFH) was also analysed. The cells displayed FXIII-A positivity in MFH, thus these cells can be separated from the histologically similar soft tissue tumors. It was found that the FXIII-A positivity did not appear in malignant cells, only in tumor-associated cells, like macrophages, follicular dendritic reticulum cells (DRCs),

fibroblast-like mesenchyme, as well as sinusoidal and interfollicular histiocytic reticulum cells.

It was supposed that malignant megakaryocytic and monocytic/cytoid cells may also express FXIII-A. Invernizzi and coworkers examined samples from patients suffering from acute myeloid leukaemia (AML), using anti-FXIII-A rabbit antibody with the peroxidase anti-peroxidase (PAP) immunological method. In the megakaryocytic (M7), myelomonocytic (M4), and monocytic (M5) subtypes a positive reaction was detected.

In our studies, a monoclonal anti-FXIII-A antibody was first applied, to examine the expression of FXIII-A in AML samples by flow cytometry. This proved to be useful in laboratory diagnostics and monitoring of this disorder.

AIMS

My aim was to investigate cellular factor XIII in AML.

- We examined the appearance of different subunits of FXIII in normal myeloid cells and AML samples.
- The sensitivity and specificity of FXIII-A expression were tested in these conditions.
- FXIII-A positivity was studied as a leukemia associated immunophenotype (LAIP) and its role in the differential diagnosis of AML was also established.
- The possible prognostic role of FXIII-A positivity in acute promyelocytic leukemia (APL) was investigated.

MATERIALS AND METHODS

Clinical and Control Samples

We examined bone marrow and peripheral blood samples in AML patients. Between the years 2000 and 2010, we carried out a systematic flow cytometric analysis of FXIII-A expression in 100 patients who suffered from AML. Six samples of peripheral blood from patients with chronic myelomonocytic leukemia (CMMoL) were also studied. All bone marrow samples underwent a morphological, cytochemical, and immunophenotypic evaluation.

We particularly focused on the promyelotic subtype of acute myeloid leukemia (AML M3), where we analysed 14 *de novo* diagnosed acute promyelocytic leukemic (APL) bone marrow and peripheral blood specimens. In these cases a blast ratio over 70% was described by morphology. Each APL belonged to the hypergranular type and by FISH technique the typical 't(15;17)' was detectable. To study normal promyelocytes, samples from bone marrow were obtained from 4 patients suffering from iron-deficiency anemia. All leukemia samples were obtained from *de novo* identified patients before any treatment.

Immunophenotype identified by flow cytometry

During immunophenotyping we investigated intracellular markers and surface markers by direct-labeled monoclonal antibodies in 3-7-color panels. The monoclonal antibodies, against the FXIII subunits, were conjugated with FITC.

Most measurements were executed on a FACSCalibur flow cytometer (Becton Dickinson), via applying the same PMT setting for each sample. In case of *de novo* leukemias, the data of 10000 cells were gained in 'List-mode files' and were analysed by Cell Quest 3.2 software. The expression of FXIII-A was also examined on normal bone marrow promyelocytes. These measurements were carried out on a 'FACSCanto II' flow cytometer equipped with 3 lasers.

We analysed the percentage of positive cells within the tested cell population and we also considered the mean fluorescence intensity (MFI) of the positive cells, that could better characterize certain malignant cell types.

Cytogenetics and FISH

Conventional cytogenetic analysis was performed on bone marrow samples in all APL patients. The karyotypes were described according to the International System of Human

Cytogenetic Nomenclature (ISCN, 2009). Fluorescence *in situ* hybridization was carried out on cell suspensions from chromosome preparation according to the manufacturer's instructions using PML-RARA dual color, single fusion translocation probe. Cells were counterstained with DAPI. In general, 200 interphase cells were counted in each case. The images were captured by Zeiss Axioplan2 fluorescence microscope and analysed by ISIS software.

Conjugation of FXIII Antibodies with FITC

The conjunction of the antibodies to FXIII was performed by the Fluoro Tag antibody labeling kit (Sigma, St. Louis, MO, USA). Bound and free forms of FITC were separated by gel-filtration.

Cell Cultures

The Mono Mac6 were a gift from Dr. Ernő Duda (Biological Research Center, Szeged, Hungary). The 'PLB-985' cell line was obtained from the Institution of Haematology and Immunology (National Medical Center, Budapest, Hungary). Monocyte maturation was achieved by 30 nM vitamine D_3, while granulocyte maturation was stimulated by 1 μmol all-trans retinoic acid and 1.25% dimethyl-sulfoxide induced. Cell surface and cytoplasmic markers were analysed up to three days.

ELISA Assay

Detection of FXIII-A in cell lysates from APL samples was carried out as described earlier, by Katona et al with a slight modification. To avoid platelet contamination, promyelocytic cells were washed three times in PBS containing EDTA. To inhibit serine and cysteine proteases, a protease inhibitor cocktail was added to the washing buffer. The exact cell count was measured before sonication in order to calculate the amount of FXIII-A/cell.

FXIII-A detection by Western Blot

After determining the FXIII-A content of the platelet-free APL blast cells, remaining cells were centrifuged and dissolved in 100 μL of SDS PAGE sample buffer. Subsequently, the denatured cell suspension was boiled for 5 minutes. Samples were loaded onto 7.5% SDS polyacrylamide gel and electrophoresed under reducing conditions. Western blotting was performed using Immobilon P membrane. Sheep polyclonal anti-human FXIII-A antibody was applied as primary antibody. The immunoreaction was developed by biotinylated rabbit

anti-sheep IgG and avidin-biotinylated peroxidase complex and visualized by enhanced chemiluminescence. FXIII-A cleavage was studied after incubation with purified human neutrophil elastase (HNE). Samples were incubated in the presence of Ca^{2+} up to 3 hours at 37 °C. The reaction was terminated by adding equal amount of SDS Laemmli buffer. The platelet-free promyelocyte cell lysates were purified from the peripheral blood of two patients and from one bone marrow sample.

Confocal Laser Scanning Microscopy

Cytospin preparations were prepared of samples derived from APL patients. The cells in the buffer containing FITC-conjugated anti-FXIII-A monoclonal antibodies, BSA and Triton X-100 were incubated for 30 minutes at room temperature, allowing permeabilization of the cell membrane. During the last 5 minutes of incubation propidium iodide (PI) was added to the labeling solution. Finally the samples were mounted with Mowiol. A Zeiss (Göttingen, Germany) LSM 510 systems and a CApochromat 63x/1.25 NA water immersion objective were used for analyses.

Statistical Analysis

The data showed a normal (Gaussian) distribution during the comparison of the ratio of positive cells found in AML subtypes, therefore we used the Student's paired t-test. Since non-normal distribution was shown in MFI values of positive cells in normal and leukaemic samples, the Mann–Whitney U test was used for statistical evaluation. The statistical comparison of the survival time in FXIII positive and FXIII negative populations was performed by GraphPad Prism 4.0 software.

RESULTS

FXIII-A expression in normal bone marrow and peripheral blood samples

After surface staining cells in the myeloid gates were analysed. Monocytes expressed the characteristic CD14, but no positive reaction was obtained for any of the FXIII subunits by surface labeling. After permeabilization with Intrastain, all myeloid cells showed myeloperoxidase (MPO) positivity. Cells of the monocytic group appeared as a typical MPO-dim population and in these cells FXIII-A was also detectable, while FXIII-B staining was completely negative. The polymorphonuclear granulocytes were MPO-bright and these cells were negative for both FXIII subunits. Lymphocytes did not show any surface or intracellular FXIII-A positivity.

In normal bone marrow samples FXIII-A could be detected in the myeloid gate in two cell populations (i) on mature CD45-bright monocytes and (ii) on CD45-dim monoblasts. Our results demonstrate that only the 'A' subunit of FXIII is present in normal peripheral blood and bone marrow intracellularly in the monocytic lineage.

FXIII-A staining specificity and sensitivity in cell lines

In cell cultures we examined the expression of the myeloid markers: CD33 and cyMPO, the monocyte markers: CD64 and CD14, the ganulocyte marker CD11b as well as the expression of FXIII-A. FXIII-A was already detectable in the Mono-Mac6 monoblast cell line on day 0, and displayed a constant intense positivity throughout 3 days of culturing, similarly to CD33. The CD14 marker was not expressed in the early stage of differentiation, but gradually became positive upon vitamin D treatment.

In contrast to CD14 expression, the intensity of FXIII-A expression, was already high also in the early stages of monocyte maturation, so FXIII-A seems an early and more sensitive marker in identifying monocytic cells. In the 'PBL-985' cell line, there was no FXIII-A expression and it remained negative throughout granulocytic differentiation that we monitored by CD11b staining.

FXIII-A labeling of AML samples

The analysis of FXIII-A expression was performed on 100 bone marrow and peripherical blood samples obtained from AML patients, earlier by 3-colour and later by 4-color labeling. In addition, we studied 6 periheral blood samples of CMMoL patients. Myeloblastic (M0, M1, M2) and erythroblastic (M6) AML did not express FXIII-A, or the degree of labeling

was insignificant. In AML M4 and M5 cases, however the ratio of FXIII-A positivity - defined as the 20% cut off - was above, the positivity rate of the CD14 surface marker referring to a better sensitvity of intracytoplasmic FXIII-A than surface CD14 in these AML cases.

FXIII-A and CD14 staining in AML and in CMMoL samples

We compared the FXIII-A and CD14 positivities in different AML subtypes. We did not observe FXIII-A expression in AML M0, M1 and M2. In the M4 and M5 groups the mean proportion of FXIII-A positive cells was considerably higher than CD14 positive cells ($p<0.0001$). FXIII-A expression was particularly high in M5a cases (mean FXIII-A positivity=68%) where the majority of cells was immature.

In the 6 CMMoL patients, the percentage of FXIII-A positive cells in FXIII-A and CD14 positive cells were exactly the same. We concluded that in mature monocytes both markers were expressed to the same extent. Comparing myeloblastic and monoblastic leukemias, FXIII-A was 100% sensitive and 95% specific for subtypes with monocytic involvement (AML M4 and M5).

We compared MFI values of normal monocytes with samples containing leukemic monocytes (AML M4, M5, CMMoL). We found that FXIII-A expression was more intense in the leukemic cells than in the normal monocytes ($p<0.05$).

In the AML group, we found FXIII-A positivity in some AML M3 patients. This was an unexpected result, because no FXIII-A expression was noticable in the neutrophil precursor cells. We then, executed a systematic examination on each *de novo* acute promyelocytic leukemia and verified the existence of this LAIP by various methods.

Flow Cytometry of AML M3 leukemias

Leukemic promyelocytes are large cells that possess numerous granules in the cytoplasm, thus are characterized with a high side-scatter and enhanced autofluorescence. These characteristics were also observed in samples from our patients.

On these APL samples we studied the expression of myeloid markers (MPO, CD13, CD33, CD14, CD15), blast markers (CD34, CD117), HLA-DR and FXIII-A.

The CD45-dim leukemic promyelocytes expressed MPO and CD33 markers intensively. The CD117 blast marker could be detected on leukemic promyelocytes, but they were negative for CD34 and these CD117-dim cells did not express the CD15 granulocyte marker. These CD33-bright, MPO-bright, and CD117-dim cells also expressed FXIII-A.

FXIII-A detection in APL Cells by CLSM

In order to investigate the intracellular localization of FXIII-A in leukemic promyelocytes, three FXIII-A positive APL samples were analyzed further by CLSM. A considerable percentage of these cells showed FXIII-A positivity. The FXIII-A protein was localized in the cytoplasm of the malignant promyelocytes.

Immunophenotypes of AML M3

The value of marker expression was considered to be positive if at least 30% of cells stained positive. The cells in all 14 samples were MPO and CD33 positive (83% and 90% respectively) while the ratio of CD13 positivity was somewhat lower (59%). All but one sample were positive for CD117 and one of the 14 APL cases also showed CD15 expression. As expected, none of the APL cases expressed HLA-DR or CD34.

Ten samples out of 14 APL cases displayed FXIII-A positivity, that considerably exceeded the 30% limit, while FXIII-A labeling did not reach the cut-off value in the remaining 4 samples

FXIII-A expression of normal promyelocytes

We analysed normal bone marrow samples by a 7-colour labelling on a flow cytometer. The normal promyelocytes, which were CD15, CD33, CD117-dim positive and HLA-DR negative and did not express any FXIII-A marker. The results revealed that FXIII-A can only be detected in malignant leukemic cells of APL, but not in normal promyelocytes.

Detection of FXIII-A by ELISA and immunoblotting

Western blot analysis with a highly sensitive chemiluminescent developing system was utilized to detect FXIII-A antigen in blast cells. We investigated bone marrow and peripheral blood samples. A single FXIII-A positive band at 82 kD was detected in APL blasts that comigrated with FXIII-A in the positive control, i.e. the platelet lysate,

Since several additional bands of smaller molecular weight were also observed, and since promyelocytes contain a lot of elastase, we compared these bands from the promyelocytic cell lysates to the FXIII-A cleaved products treated with purified human neutrophil elastase and the staining patterns showed a high degree of concordance.

In case of two samples we measured the quantity of FXIII-A antigen in the cell lysates by ELISA method. These specimens contained more than 90% of promyelocytes and no monocytes were detectable. In these two samples we measured a value of 29 and 80 fg/cell. The antigen quantity of these promyelocytic leukemia cells, was similar as previously defined for platelets: 60±10 fg/platelet. These results also showed a correlation with the values of MFI, determined by flow cytometry.

Survival in APL based on FXIII-A expression

We found FXIII-A expression in the malignant promyelocytes in 10 out of 14 cases. Monitoring the patients' survival times, it turned out that there was a significant difference between these groups ($p<0.0001$, with logrank test). The FXIII-A-negative patients did not react well to treatment or relapsed quite soon. Their median survival time was only one month, in contrast to the FXIII-A-positive patients - followed for up to 5 years - who are still alive.

DISCUSSION

Flow cytometry was a huge step in the diagnostics of leukemias, and nowadays it serves as a fundamental method in the differentiation of leukemia subtypes. In this study, we analysed the immunophenotypes of the cells by the detection of intracellular and surface markers in a multicolor setting.

Morphological and cytochemical methods can evaluate much less cells than flow cytometry. In *de novo* cases, 10-50 x 10^3 cells are routinely analysed, while in follow-up studies data of hundreds of thousands of cells are collected, when the number of residual cells should be determined. Flow cytometry services started with 2-3 colors and nowadays, in larger centers it is carried out with 6-10 colors. The major aim is to to define the cell line, that can be achieved most reliably by detecting the intracytoplasmic markers (cyCD3 - T-cell, cyCD79α - B-cell, MPO - myeloid cells). Moreover, the intracytoplasmic markers can also provide some useful information about cell maturity. By the multiclor examination of these surface and intracellular markers, LAIP can be identified.

Acute lymphoblastic leukemia (ALL) was the first hemtological malignancy investigated in detail by flow cytometry. AML phenotyping is a more recent entity, where the identification of the myeloid origin, is established on the intracytoplasmic presence of the MPO marker. After identifying the cell line, further intracytoplasmic and surface markers are used to establish cell maturation and the AML subtype.

Morphological features and cytochemical reactions provided the basis for the FAB (French-American-British) classification. Accordingly, the following AML subgroups exist: M0-M4, M5a, M5b, M6-7. (M0: with minimal differentiation; M1: without maturation; M2: with maturation; M3: acute promyelocytic leukemia; M4: acute myelomonocytic leukemia; M5a, b: acute monoblastic, monocytic leukemia, M6: erythroid leukemia M7: acute megakaryoblastic leukemia.)

As the prognosis and the treatment of AML subtypes may be different it is imporatnt to know the exact AML subtype. This categorizing is done by the evaluation of the marker-specific expression patterns of the malignant cells.

In recent WHO documents the AML classification is based on genetic abnormalities. According to its 2001 issue, 50-60% of AML cases belonged to a so-called AML-NOS category (NOS= not otherwise specified). In those cases normal karyotypes were found. In the 2008 WHO classification, three new chromosomal changes were incorporated as follows: t(6;9), inv(3), t(1;22). These are rare abnormalities, but their identification is important,

because they are associated with poor prognosis and they require allogenic stem cell transplantation. Moreover, two new, provisional entities were established: AML *NPM1* (nucleophosmin) and AML *CEBPA* (CCAT/enhancer binding protein alpha) with mutation. Both are often accompained with normal karyotypes. These new markers reduced the AML-NOS category to 30%. In the NOS category the scaling is similar to the old FAB classification.

In spite of the fact that genetic differences provide the basis for AML classification, morphological examination and immunophenotyping remained a frontline examination method. Subtyping may be difficult in several cases e.g. the diagnosis based upon the lack of markers in M3, the verification or precluding of monoblast origin and separation M0-M2 types from M4-M5. The M0-M2 and M4-M5 leukemias may be differenciated by the expression of CD14 monocytic marker. It was previously known that cells of monocytic cell lines contain FXIII-A protein. The assumption, that FXIII-A can be detected in malignantly transformed monoblasts/monocytes, was verified by immunomorphological methods.

For the first time we used multicolor flow cytometry to detect the presence of FXIII-A in AML bone marrow and peripheral blood samples. In accordance with the early descriptions, only the ‘A’subunit is present in the peripheral blood and bone marrow cells. FXIII-A positivity was detected only after the permeabilization, so it must be intracellular.

We did not identify any FXIII-A in granulocytes, only in the MPO-dim, CD14 positive monocytic cells. In bone marrow samples we obtained positivity in the CD45-bright monocytes and in CD45-dim monoblasts. This means, that FXIII-A is an ideal marker to separate granulocytic and monocytic cells.

We examined the timely appearance of FXIII-A on cell lines of granulocytic and monocytic differentiation. FXIII-A did not appear during granulocytic differentiation, however, during monocytic differentiation we identified FXIII-A even at the most immature stages with high intensity, while the CD14 surface marker was only detectable upon differentiation. This identifies XIII-A as a very early marker of the monocytic cell line when the CD14 marker has not yet been expressed on the cell surface.

Based on these results we investigated FXIII-A positivity in AML specimens by flow cytometry. In the subtypes of M0, M1, M2, M6, no positivity was found with only one exception. This FXIII-A positive AML case aside the monocytic and megakaryocytic subtypes proved to be M0. In this case the FXIII-A positivity is not very surprising, because these malignant, undifferenciated blasts may often express at the same time myeloid, B-cell or T-cell markers. In the M4 and M5 AML cases FXIII-A expression exceeded that of CD14

labeling. Analysing MFI values, it could also be concluded that FXIII-A differs from other intracytoplasmic markers, since it is present in malignant cells with higher intensity than in the normal cell populations. In M4 and M5 AML cases and in CMMoL patients FXIII-A MFI values were significantly higher than in normal monocytes.

FXIII-A is also a useful marker in the classification of AML M7, since its presence has been described in the megakaryocyte cell lineage and a very strong FXIII-A fluorescence was detectable in malignant megakaryocytes.

An unusual pattern of marker coexpression in acute leukemias also referred to as, the LAIP, gained great importance, since when looking for minimal residual disease, this enables the detection of malignant cell populations from subsequent samples. LAIP aids in the differentiation of leukemic blasts from normal hematopoietic progenitors in the regenerating marrow.

Aberrant immunophenotypes can manifest in several forms, e.g. bilinearity, cross-lineage antigen expresson, antigen overexpression, asynchronous antigen expression or aberrant light-scattering.

The importance of LAIP is now increasingly been recognized also in connection with AML. Although from the prognostic point of view, the cytogenetic abnormalities are the most acceptable independent risk factor, it is known that in nearly half of AML cases there is no chromosomal abberation. Several retrospective analyses were carried out in which the marker expression patterns of several hundreds of AML patients were compared to therapy response, the ratio of complete remission and relapse. The results were miscellaneuos and the relation between the myleoid marker (CD13, CD33, CD117) expression and the prognosis was not evident.

Even today the outcome of an AML in the adulthood is relatively poor. Only 25% of the cases reaches 5 years in survival time (www.seer.cancer.gov). Since there is no cytogenetic abnormality in many cases, the study of other alternative prognostical factors came in the foreground. Two transmembrane proteins causing chemotherapy resistance were widely studied; the P-glycoprotein and the FLT3 tyrosine kinase. The inefficiency of the chemotherapy related to these proteins means a significantly shorter survival time.

In our early studies we also identified an AML M3 case with FXIII-positivity, that was a surprsing result, that is why we looked systematically at further APL samples. FXIII-A antigen was found in 70% of promyelocytic leukemic cells. The leukemic cells showed a typical pattern based on lack of markers (CD34-/CD15- and HLA-DR negativity). The CD45-dim cells had an intensive autofluorescence and MPO, CD33 and CD117-dim positivity. The

presence of FXIII-A in the cells was also confirmed by CLSM, where an intracytoplasmic localisation was detected.

By a sensitive ELISA we found, a high FXIII-A content in malignant promyleocytes its quantity was similar to the FXIII-A content of platelets and 10 times more than in another LAIP associated expression in leukemic lymphoblasts. The structure of the subunit 'A' within the cell was examined in platelets and was found in A_2 dimer form, similarly to other intracellular forms. The process of FXIII-A activation differs in case of plasma and in the cytoplasm. In the plasma, thrombin cleaves the activating peptide in the presence of Ca^{2+} and the active enzyme (FXIIIa) is formed. Cellular activation is, nevertheless, a much slower process. No proteolysis is required and the activation is started under the effect of calcium. We do not know any mechanism that controls the process of inactivation, however, it has been recently proved, that human neutrophil elastase (HNE) plays a role.

In the APL-specimens we evaluated the forms of FXIII-A protein by Western blotting. On the Western blot images we detected a band at 82 kD originating from the patients' promyleocytic cell lysates, referring to FXIII-A. Several more bands were detected, as well, referring to some other smaller proteins. Comparing the bands obtained by the cleavage products with the ones received from purified FXIII-A_2 cleaved through proteolysis by HNE, we got a similar pattern. Thus, we concluded that the smaller mass fragments, originating from FXIII-A found in APL samples are probably formed through the intracellular proteolysis by HNE.

Studies carried out on cell lines supported the idea that cells of myeloblast origin express no FXIII-A antigen in any phase of the cell differentiation. Examining normal promyelocytes we also confirmed this notion, since FXIII-A can not be detected in normal promyleocytes. From these data, we could conclude that FXIII-A expression in leukemic promyelcytes is LAIP. APL, according to the 2008 WHO-classification, is an independent entity with the unique genetic abnormality; t(15;17). The origin of APL cells is not entirely clear. Monocyte markers such as CD9 or CD68, but not CD14, have already been detected on APL cells. To find markers which are positive only for APL would be of great importance, because APL immunophenotyping is recently based mostly on finding marker negativities. Due to the low number of our APL cases, we can not judge, whether there is a connection between FXIII-A expression and the presence of disseminated intravascular coagulation. Bleeding can be the consequence of diverse reasons. However, neutrophil elastase may also contribute to it. We observed that in those who cases who did not react to treatment or in

whom a relapse soon occured, no FXIII-A reaction could be detected. In their case the mean survival time was about one month. To date, all FXIII-A positive APL patients are all alive.

According to our results, examination of FXIII-A marker is useful in solving differential diagnostic problems in AML and searching for MRD. In case of AML M3 the FXIII-A marker indicates leukemia-associated immunophenotype (LAIP), which also have an effect on the prognosis.

SUMMARY

FXIII of blood coagulation is a protransglutaminase, its active form stabilizes the fibrin network in the final phase of blood coagulation. In the peripheral blood it is circulating as a heterotetramer (A_2B_2) while its cellular form is a homodimer (A_2). It is present in the megakaryocytes /platelets and a monocyte/macrophage cell lines and was also detected upon malignant transformation of these cells.

In our studies we investigated the expression of Factor XIII in AML. Below is a summary of the conclusions of my studies.

- By using monoclonal antibodies to the A and B subunits of FXIII we established by flow cytometry that in normal peripheral blood and bone marrow only the A subunit is present intracellularly in cells of monocytic origin.

- By investigating relevant cell lines we found that during granulocytic differentiation, FXIII does not appear at any stage, but is present during monocyte maturation even in the most immature cells and CD14 appears only in the more mature forms. During maturation intracellular markers appear earlier than surface markers.

- In M4 (myelomonocytic) and M5 (monoblastic and monocytic) AML subtypes, cells of the monocytic lineage could be identified based on their FXIII-A content while the surface CD14 was still negative.

- During our studies we identified FXIII-A antigen in APL cells. The enzyme was localized in the cytoplasm and beside the intact protein, fragmented forms were also identified by Western-blotting.

- FXIII-A was not expressed in normal promyelycytes, thus can be regarded as a leukemia associated immunophenotype. Formally the diagnostics of APL was based on negativity of certain markers but FXIII-A positivity can be used in the differential diagnostics.

- Based on the limited number of investigated APL patients, a favourable survival of FXIII-A positive cases is suggested, that requires further studies.

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List of publications related to the dissertation

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