Ph.D Thesis

Complex haemostaseological and molecular biological examination of diseases with abnormal platelet-count or function

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Introduction

Abnormal platelet function can be caused by different diseases and conditions. The altered platelet-function may later play a role in the progress and pathogenesis of the underlying disease. In other cases, like in chronic myeloproliferative diseases (CMPD) the very high platelet-counts may seem to be the most important factor of complications (either haemorragic, or thrombotic). Pathological platelet function (acquired thrombocytopathy) can be demonstrated in most cases even so. Moreover the clinical profile can imply this.

In some diseases and conditions (like myeloproliferative diseases with high platelet-count, reactive thrombocytoses, diabetes mellitus, and posttransplantational diabetes mellitus) essential practical consequences can be inferred from examinations of supposed, or proved cases of haemostasis abnormalities besides the theoretical importance. Genetic examination of diseases with high platelet-counts, as well as myeloproliferative, and other proliferative haematological diseases can also have important practical significance: besides their role in setting up diagnosis, and differential-diagnosis, their application in monitoring therapy and following up patients can not be disputed.

Various laboratory methods are mentioned in the literature for the investigation of conditions with high platelet-counts, which may help differentiate between primary and secondary thrombocytosis. None of them, however, are recommended as being reliable enough to be applied as a screening test. The shear-induced platelet-aggregation (using PFA, or O'Brien filterometer) examination could serve as a new possibility to differentiate between these two completely different conditions. In the present Ph.D. thesis I will analyse the possible role and diagnostic advantage of the O'Brien filterometer as compared to previously applied laboratory methods. We were also able to run experiments of further essential parameters (NO-production, lately F XIII) of haemostasis. We have examined in vivo ß-thromboglobulin, which indicates platelet activity and plasma-levels of other important haemostatis parameters (e.g. soluble thrombomoduline), in patients with diabetes mellitus, and with diabetes mellitus induced by immunosuppressive therapy following kidney transplantation. We have examined the correlations between individual laboratory values and relevant laboratory and clinical data. Finally, in Hungary I have been among the first few to acquire some hands on experience in the applicability of certain molecular biological (e.g. FISH) examinations in myeloproliferative diseases, mostly in CML. We have

of course used these examinations in other malignant, haematological diseases as well. The latter examinations provide supplementary data to ongoing research aimed at finding more effective treatment and better follow-up of some haematological malignancies.

Accordingly the present thesis is divided into a few sub-chapters, but basically into two main units:

firstly, the haemostasis examination of diseases and conditions with high platelet-count, or abnormal platelet-function, and

secondly, molecular biological examination of myeloproliferative diseases (therefore characterized by abnormal platelet-function) and other proliferative haematological diseases.

Aims

In the course of my research I wanted to find answers to the following questions:

1) What is the diagnostic value and use of the shear-induced platelet-aggregation (using the O'Brien filterometer) in conditions with high platelet-counts? Can it be used as a simple and fast screening test to differentiate between primary and secondary thrombocytoses? What issues can be raised based upon the present results, which would initiate further examinations?

2) In the middle of the 1990-ies it was revealed that thrombocytes also have the NO synthase enzyme capacitating EDRF-NO (NO) production. The small amount of NO produced in the platelets decreases the number of aggregated platelets. The disturbance of thrombocyte NO generation can theoretically play a role in thrombotic complications of chronic myeloproliferative diseases with elevated platelet-count, but experimental data supporting this theory is not yet known. Our next aim therefore was the quantitative definition of NO produced in thrombocytes.

3) Haemostasis disorders are supposed to play an important role in posttransplantational diabetes, contributing to a higher frequency of cardiovascular

morbidity and mortality. Therefore it seemed to be reasonable to test, whether hyperactivity of platelets and damage of the vascular endothelium could be proved in patients who developed diabetes as a consequence of immunosuppressive treatment following a successful kidney transplantation. Examination of PAI-I activity, as well as production of intravascular EDRF-NO may also be of significance, as suggested in various publications.

4) Classical tumour cytogenetics is a valuable method to explore chromosome anomalies in malignant diseases. However, a subpopulation of malignant cells may sometimes be selected in the cell cultures, during cytogenetics which is not necessarily relevant of the tumour itself, (but has some proliferative advantage in the given circumstances). The number of metaphases available for the assay is also limited. We have decided to use the fluorescence in situ hybridisation (FISH) as well as comparative genomial hybridisation (CGH) methods, which have just become available to answer the following questions:

Do the results of an additional FISH/CGH examination provide more information than the classical cytogenetic test in malignant haematological diseases and thus help set up a better diagnosis and achieve a better mapping of the genetic disorders?

What is the value of FISH tests compare to conventional cytogenetic tests? Should it be incorporated into the complex diagnostic protocol of leukaemia patients? How much would the FISH test of bcr/abl gene translocation (Philadelphia-chromosome, like marker-chromosome) help verify or exclude the diagnosis or follow the progress of disease in case of a supposed CML?

Can FISH tests help assess the prognosis of biological behaviour of diseases, i.e. help choose the most appropriate treatment strategy (chemotherapy, need for early transplantation)?

Can peripheral blood samples be used with the same effectivity as the traditionally used bone-marrow samples?

Patients and methods

1. In order to differentiate primary thrombocytosis from secondary we have used the less known O'Brien filterometer on ***"shear-dependent" platelet-aggregation***. We investigated 53 patients suffering from chronic myeloproliferative disease There were 27 males and 26 women in the group, with a mean age of 56 (range: 21-89). Reactive throbocytosis was detected in 21 cases (9 males, 12 females) with a mean age of 52 (range: 18-88).

The control group consisted of twenty healthy volunteers (9 males, 11 females). Their mean age was 31 (range: 20-50).

We used the O'Brien filterometer to evaluate the "shear-dependent" platelet aggregation. Whole blood anticoagulated with heparin and citrate was forced at a pressure of 40 mmHg through a filter. Under these conditions platelets become activated and aggregated. The aggregates then block the filter. The number of drops going through the filter is recorded every 5 seconds. Drops were collected and counted before the filtering, in the first 5 and between 20-40 seconds.

Characteristic parameters:

-*blocking drop count*: the count after which no more than one drop at most can pass the filter in the next 5 seconds. (In case of heparin- or citrate-anticoagulated blood of healthy controls the blocking drop count was 11-29; 11-42 respectively)

-*platelet retention* (R): it is calculated from the number of platelets in the drops between the first 5 seconds (R-I) and between 20-40 seconds (R-II) and pre-filter platelet count. (Normal values for R-I using heparin or citrate are 33-98% and 45-89% respectively, whereas normal values for R-II they were 85-100% and 68-100% respectively.

Bleeding time was always measured, as well as Willebrand-protein plasma level (vWF:Ag) by ELISA method.

2. ***Examination of platelet NO production*** was analysed by using washed platelets of both healthy people, and patients with high platelet count due to chronic myeloproliferative disease. We examined 15 patients with myeloproliferative disease There were 10 males and 5 females in the group with a mean age of 65.4 (range 38-80). Platelet count was in all cases abnormally high (above $500x10^9$/L).

The control group was made up of twenty healthy volunteers (7 males and 13 females) with a mean age of 39.8 (range: 29-54). None of the members of either group received medication with nitrite content.

Sampling: venous, anticoagulated blood (ACD [citrate]-solution, Beckton-Dickinson) was centrifuged at 200 g for 10 minutes, then washed three times in HEPES buffer (pH 7.4), and finally after the third wash platelets were resuspended in 0.9% NaCl solution. The platelet count was set as standard $100x10^9$/L.

Measurement of NO production in the platelet-suspension: Measurements were done with the help of NO-sensitive microelectrode (ISONOP 200, ISO-NO WPI, Sarasota, Florida). To standardize NO quantity we used the SNAP donor attached to the equipment by the manufacturer, at six different concentrations. Non-activated platelet NO production was determined in L-argin buffer and taken as baseline. Then activators were added: collagen (2.5 mg/ml), ADP (1.0 μg/ml), thrombin (3.1 mU/ml), adrenalin (0.25 μg/ml) and ristomycine (31,5 μg/ml). Bracketed numbers stand for the final concentration of activator in the tube.

We did the examinations in all cases with the L-argin competitive metabolic inhibitors (from which no NO production is possible). For this we applied L-NAME (nitro-L-arginine-methylesther), and L-NNA (L-nitro-L-arginine).

Platelet NO production resulted in an electrochemical response of the pikoAmper (pA) range in our platelet-activator system. The initial sharp rise, followed by a plateau phase were plotted against reaction time as a curve, and characterized by well known enzyme-chemical parameters, thus: Vmax: pA against time, Km: the time to achieve half of maximum response. Vmax seems to be suitable to quantify platelet NO production (these were fit to SNAP standards), whereas Km gives information about the speed of production. (Higher Km value indicating slower production).

3. *Laboratory tests in* posttransplantational *diabetes mellitus*

We measured the activity of the serum plasminogene-activator inhibitor PAI-1, the level of tissue-type plasminogene activator tPA, (using enzyme chromogene reaction, in 47 patients), ß-TG, which reflects platelet activity (by using ELISA in 53 cases) and sTM, which is released due to the erosion of the endothelium, as well as endothelial NO production, and total antioxidant status postoperatively in 53 kidney transplant patients, (operated at Clinic of Transplantational Surgery, in Budapest) who developed diabetes after having received immunosuppressive therapy (steroid + cyclosporin A ± azathioprin ±

mofetilium mycophenolicum). There were 34 men and 19 women in the group with a mean age of 47.6 (range 14-68). The mean postoperative period was 54.8 (range 2-151) months.

The control group consisted of 29 healthy people (16 males, 13 females) with a mean age of 42.8 (range: 31-65).

NO production of the endothelium

NO is a molecule, with a very short half-life (3-5 seconds), which is soon transformed into NO_2, and NO_3. Although direct measurement of endothelial No production is rather difficult, the production is reflected in the nitrite/nitrate content of the serum/urine, which is easier to measure. (The nitrate in the serum and urine can be reduced to nitrite by using nitrate reductase. The nitrite present before the reduction and the nitrite produced via nitrate reduction can be defined with the help of the Griess reaction). Thus we can examine the intravascular EDRF-NO production in a relatively simple, non-invasive way.

Total antioxidant status (TAS)

We measured this with the "Total antioxidant status" kit (Randox, Wales, UK).

Measurements of HgA1-C, urea, creatinine, cholesterine and triglyceride were carried out in the Central Laboratory of the Clinic of Transplantational Surgery.

4a. Comparative Genomic Hybridisation (CGH)

We managed to perform a CGH examination on five patients (CLL:3; CML:1; ALL:1).

The CGH analysis worked out by Kallioneimi et al., was carried out according to previous descriptions. The DNA needed for hybridisation was taken from leukaemia cells, and normal peripheral lymphocytes, then, prepared with "Genomic DNA Purification Kit" as defined in the instructions supplied with the kit. The DNA from the leukaemia cells were stained with nick-translation (green fluorescent) Spectrum Green-12-dUTP, the normal DNA with Spectrum Red-5-dUTP (red fluorescent) according to the protocol of the manufacturer (Vysis, Inc. Downers Grove, IL USA). For the nick translation we used the "Vysis, CGH Nick Translation kit". The hybridisation compound contained equal amounts of leukaemia and normal (marked) DNA. Denaturation of the DNA (at 73 C for 5 minutes) followed. The target (in metaphase) chromosomes (Vysis Inc.) of healthy males were denaturated for 3-5 minutes at 73 C. The hybridisation compound was the dropped on a slide and hermetically sealed. The period of hybridisation was 72 hours. During this time the preparations were kept in a wet chamber at 37 C. After hybridisation we removed the non-hybridised DNA

from the slides. The nuclei were stained with blue DAPI dissolved in anti-fade solution (Vysis Inc.)

Digital Image Analysis: The three fluorescence intensities were taken with a CCD camera (Compulog, IMAC-CCD-230, 8 bit, Metasytem GmbH) using green, red and blue optical filters. (It is advisable to examine 8-10 metaphases per sample). The image analysing system is based on the Zeiss Axioplan 135 microscope (Carl Zeiss, Jena, Germany) to which a computer-operated quantitative image analysing system was attached. The automatic colour kariotype analysing program using the image visualised by DAPI can carry out the identification of the chromosomes. The green fluorescence originates from hybridised leukaemia DNA, the red fluorescence from hybridised normal DNA. To determine the chromosome-deviances the ratio of the quotients of the green/red fluorescence intensity can be used. Calculating the mean of chromosome profiles related to the individual metaphases we can get information about mean differences typical of the given disease. Excess DNA can be observed if the ratio of the green/red fluorescence intensity is more than 1.15, whereas in case of DNA loss the ratio is less than 0.85.

4b <u>Fluorescence in situ hybridisation (FISH)</u>

The FISH-tests were carried out on lymphocytes originating from bone marrow and/or peripheral blood samples of patients treated at the Second Internal Clinic of DEOEC. After proper preparation the cells were dropped on slides, and stored at –20 until FISH-test was carried out.

Before hybridisation the preparation on the slide was denaturated (for 5 minutes, at 73 C on an appropriate pH.). Under these conditions the double helix of the DNA structure can be separated, and the denaturated DNA-probes, labelled with fluorescent stain, containing complement sequences were hybridised on these. (The probes represent a small genome part). After hybridisation, and removal of the superfluous probe in a series of steps, the nuclei were stained with blue DAPI, a DNA-specific stain. Both the chromosomes and the nucleus then appear blue. The hybridised DNA-probe, marked with fluorescent blue stain can be made visible, and examined under an appropriate microscope.

For the FISH tests DNA locus-, and centromere-specific probes were used. With the help of the latter numerical aberrations of chromosomes can be examined. The probes hybridised to the centromere regions of the chromosomes show the number of chromosomes examined in the cells (their diploid nature, or the presence of a trisomy) by way of a big

fluorescent signal. Locus- or sequence-specific probes are applied to examine delitions, amplifications, or translocations. They facilitate identification of cytogenetic deviations even in interphase cells, and so aberrations non-definable by cytogenetic examinations can be revealed.

Most frequently we examined the presence of the Philadelphia (Ph) chromosome, i.e. t(9;22) (q34;q11.2): the oncogene c-ABL localised in the q34 region of the chromosome 9 will be translocated to the BCR gene of the chromosome 22. Thus a fused chimera gene is produced. We label the BCR locus on the chromosome 22 with a green, and the ABL locus with a red, fluorescent DNA probe. In normal cells these probes will present far from each other as individual signals. If a Ph chromosome is present, due to translocation of the chromosome segments, the two probes will appear next to each other, a partial overlap of signals will refer to translocation. The principle of examination of t(15;17) typical for acute promyelocytic leukaemia and the colour of the applied probes are the same as those used for the BCR/ABL translocation.

We carried out hybridization (FISH-test) in 82 patients (CML or suspicion of it:44; leucocytosis:4; ALL:7; AML M3(or suspicion):8; CLL:13; DMPS/AML:1) in 140 cases altogether. More than half of the patients were either suffering or suspected to be suffering from CML, or leucocytosis, justifying the examination, with which we wanted to test the presence of bcr/abl translocation, and its quantitative change. We were searching for the presence of the same translocation in ALL, and in some cases in AML too. In AML M3, or if there was the suspicion of this disease the presence of t(15;17), specific to promyelocytic leukaemia, and its quantitative change were searched for. In some cases (in AML) we also examined the numeral anomalies of some chromosomes (e.g. 1, 7, 8 etc). In CLL we mostly examined the trisomy of chromosome 12, in one case however (the disease of whom transformed from DMPS into AML) we examined the numeral anomaly of the chromosome 7. Translocations and numeral abnormalities of chromosomes were examined by direct marked locus-specific and centromere-specific Vysis probes. In some patients we examined more than one chromosome-anomaly using simultaneous hybridisation. In a few patients examinations were carried out several times, i.e. the FISH technique was used for “cytogenetic tracking”.

Results

1.Examination of primary and secondary thrombocytoses using the O'Brien filterometer:

From among the 53 patients suffering from CMPD we examined whole blood samples anticoagulated with heparin (52 cases) and anticoagulated with citrate (35 cases). Using heparin as anticoagulant the blocking drop count was abnormal in 18, the R-I in10 and the R-II in 31 cases. When examining the blood samples anticoagulated with citrate, we found abnormal values of blocking drop-count in 23, of R-I in 24 and of R-II in 27 cases.

In the reactive thrombocytosis group we made 21 tests using heparin, and 18 with citrate. When we used heparin both the blocking drop count and the R-I proved to be normal, while R-II values were false positive in 3 cases. From among the 18 examinations with citrate neither the blocking drop count nor the R-I was positive, and R-II value was only false positive in one case.

Bleeding time was abnormal only in 13 % of the patients suffering from chronic myeloproliferative disease.

When we used adrenalin, platelet aggregation was abnormal in 68 % of patients, whereas with ADP only 64 %. In case of collagene, arachidonic acid and ristomycine decreased platelet-aggregation was noticed, 36%, 23% and 28% respectively.

The mean plasma level of vWF:Ag in the myeloproliferative group was 1.19 (range 4.16-0.36) U/L, whereas in the reactive throbocytosis group the mean was 1.72 (range: 4.8-0.97) U/L.

We analysed the data, but did not find correlation between the platelet count, blocking drop count, retention I. and II and the vWFAg, i.e. there was no correlation between platelet count and vWFAg levels in either group. Significant correlation was however, found between blocking drop count and vWF-Ag levels in the reactive thrombocytosis group, when using heparin ($p=0.016$), and an even more significant correlation, when using citrate as anticoagulant ($p=0,0048$). There was however no correlation between the two values in the CMPD group ($p=0.28$ and 0.24 respectively). Significant correlation was found between R-I and vWFAg values when using heparin ($p=0.047$) in the myeloproliferative group, but the correlation was border-line significant only in reactive thrombocytosis ($p=0.064$). Quite the opposite was found with the citrate method, citrate being a more sensitive method in revealing myeloproliferative diseases, the

correlation was only significant in reactive thrombocytosis (p=0.017 vs 0.64). Analysis of R-II values and antigene levels did not yield any correlation either with the citrate method, or with heparin method.

Thus we can summarise the most important results of the filter test: the citrate method proved to be more sensitive in all cases than the heparin method. All parameters (blocking drop count, R-I, R-II) of the two groups are significantly different. The sensitivity and specificity of the R-II values of the citrate samples was 77.1% and 94.4% respectively, i.e. appropriately high to be used for examining conditions with high platelet count. The ratio of false-negative (22.9%) and false-positive (5.6%) cases is acceptable.

2. Investigation of NO-production of plateletes in chronic myeloproliferative diseases with high platelete count:

Thrombocyte NO reaction was observed within 20-40 second after the application of the activator. In the presence of competitive antagoniststs of L-arginine (L-NAME, L-NNA) NO production of platelets decreased by at least 90%.

Despite large deviation of the results the NO-production of platelets in CMD proved significantly decreased compared to control. The quantity of NO-production is activator dependent and can be effectively blocked by NO-synthesis inhibitors.

3. Examinations in posttransplantational diabetes mellitus:

In patients with posttransplantational diabetes PAI-1 activity and tPA levels were found much higher, more then twice as high as in controls. It must be noted that more and more attention has been focussed on the connection between fibrinolysis and artery diseases. A marked elevation (similar to that of PAI-1) was found in the sTM level of the plasma, which is released from eroded endothelium cells. The most striking elevation was found in the plasma level of ß-thromboglobulin reflecting in vivo platelet activation. (In this patient group the values were six times higher). Contrary to our expectations, fewer inravasal NO-production was observed in the control group.

As a result of our examinations of the correlation between haemostasis parameters and the glycosylated-hemoglobin, kidney functions, lipids, and the period after transplantation we found remarkable positive correlation in six cases: the correlation was strong, but statistically not significant between HgA1-C – thrombomodulin (p=0.0758) as well as between posttransplantational period and PAI-1 (p=0.092). However, significant correlation was found between the following values: creatinine – ßTG (p=0.01); triglyceride

– thrombomodulin (p=0.001); triglyceride – PAI-1 (p=0.028); posttransplantational time – tPA (p=0.0289).

4a. Results of CGH:

We found alteration in one patient: as a rarity, trisomy of the chromosome 7 was found, and later also proved by FISH-test. (Cytogenetic examinations favoured trisomy of chromosome 8. By using chromosome 7 and 8 cenromere-specific probes the trisomy of chromosome 7 could be proved, whereas chromosome 8 proved to be diploid.) In two more CLL, one CML, and one ALL patient we did not find kariotype-anomaly.

4b. Results of FISH tests:

We examined 44 patients with CML or suspected CML, and carried out altogether 77 hybridisations. (In case of a few patients we examined the presence of BCR/ABL translocation, or the change of the ratio of positive cells.)

Before starting INF-treatment the FISH test of 28 patients was carried out. Clinical data definitely suggested CML. In 24 cases FISH test supported the pending diagnosis of CML. The final diagnosis of the four BCR/ABL negative patients (the cytogenetic examination did not exhibit Ph chromosome either) was myelofibrosis, Ph-negative CML, neutrophil-leukaemia, and non-definable chronic myeloproliferative disease.

Due to the absence of BM aspirate we did not perform cytogenetic examination in four cases: In the first patient the positive BCR/ABL of the lymphocytes in the peripheral blood supported CML. 16 months after diagnosis the patent underwent unrelated-donor transplantation. Since transplantation the patient has been in a phase of complete haematological remission. Repeated FISH test result did not reveal any cells carrying the Ph chromosome. In patients 2 and 22 the FISH test of the peripheral blood was conclusive of the correct diagnosis, and in patient 22 it also facilitated a Glivec-treatment. The data of patient 24 (including crista-biopsy) confirmed myelofibrosis (agnogen myeloid metaplasia). Due to lack of BM aspirate, despite the severe cytopaenia, FISH test of leucocytes of venous blood confirmed BCR/ABL translocation, i.e. CML.

In four more cases FISH test implied BCR/ABL positivity, whereas cytogenetic examination did not reveal presence of the Ph chromosome.

FISH analyses were carried out on cells partly originating from bone marrow, partly from peripheral blood. Six times we carried out simultaneous examinations on both bone marrow and peripheral blood taken at the same time with similar results. Translocations affecting 100% of the cells were not present in any of the cases. From 28 patients double Ph-positivity was only present in six (a very low percentage). Small numbers must be treated

with extreme care, yet since we observed in four out of the six cases a fast acceleration, the development of a blast-phase, double Ph-positivity at the time of diagnosis (even in small percentage) could be claimed to indicate a bad prognosis, and raises the need for a more intensive treatment.

An important consequence of our examinations is, that FISH tests can be carried out on both bone marrow, and peripheral cells, as evidenced by appropriate correlation of parallel examinations implying that regular bone marrow examinations are not necessarily required. For the follow-up of patients (during treatment, to assess the change in Ph-positive cell ratio) the less strenuous examination of venous blood samples, or lymphocytes separated from the circulation could be appropriate.

From the above it follows that FISH technique can be useful, informative and of a diagnostic power in patients who for technical reasons have no BM aspirate, or if there is no possibility for a cytogenetic examination, or if its result is negative.

Conventional cytogenetic examination did not confirm Ph-positivity of patient 7. FISH test revealed BCR/ABL translocation. Later PCR tests and the clinical progress of the disease (rapidly developing blast phase) however confirmed the positive results of the FISH test. It is of some interest that our patient 8 had to be considered a Ph-negative CML case for a long time (based on negative cytogenetic and PCR tests). The FISH test however, which is less sensitive than the PCR, revealed BCR/ABL translocation. (Repeated PCR test some time later confirmed positivity.)

In a young CML patient in the blast phase we could show significant decrease of Ph-positivity following a mini ICE treatment.

For each FISH test we defined the ratio of Ph-positive cells. This is more difficult when using conventional cytogenetic tests. FISH technique could not confirm 100% frequency of BCR/ABL translocation in any of the patients with 100% Ph-positivity based on conventional cytogenetic test. This shows the more salient presence of a residual healthy haematopoesis than previously supposed.

There might be a discrepancy between the results of FISH tests and the scoring system suggested by Kantarjian et al. based on clinical parameters, which would assign patients into groups with good, intermedier and bad prognosis, similarly to the Sokal scoring system evaluating similar information. The FISH test implied bad prognosis in many cases that according to the clinical scoring system were classified as “good”, or “intermedier”. The progress of the actual disease confirmed the “bad prognosis”. The scoring system based on clinical parameters is an important element of the freshly diagnosed CML patient’s file, it is

however not as informative as the genetic test therefore the results of the latter should be given more attention.

42 FISH tests were carried out in 21 CML patients treated with INF. (The number of CML patients is not 49, but 44 because some of the patients had been included in the previous group.) Similarly to the previous group we carried out hybridisation tests of lymphocytes from both bone marrow and venous blood. There were 10 parallel examinations. The correlation of the results was good.

There was a 44 year-old female patient (first patient in chart 10 a.), who could only tolerate a low dose INF therapy (2x5 ME/week). A FISH test six years after her rapid (4 months) complete haematological remission confirmed complete cytogenetic remission. The next, a 58 year-old male patient was found to have double Ph-positivity, implying bad prognosis. Despite the complete haematological remission within 3 months, there was the need for a more intensive, combined treatment (INF+HU+ constant, low dose, s.c. Ara-C) in order to keep up this condition. In patients 3 and 4 cytogenetic deterioration, and the presence of the double Ph chromosome predicted progression and acceleration of their basic disease, the development of the blastic phase. In a patient who had undergone allogenetic bone marrow transplantation the FISH test showed persistence of Ph-positive cells, and later their increasing ratio, although there were no clinical symptoms. The continuous posttransplantational INF treatments may have contributed significantly to keeping up a good clinical condition, and suppressing the Ph-positive clone. In several cases the FISH test showed cytogenetic deterioration and therefore a Glivec treatment was started.

Similarly to the previous group the FISH test revealed the presence of the BCR/ABL translocation in Ph-negative patients too.

Double Ph-positivity proved to be more important for the prognosis again, than prediction of the scoring system based on clinical parameters.

There was one case of a possible total cytogenetic remission in the group treated with INF, based on cytogenetic results gradually turning negative, and FISH test results being within the margins of error. Partial cytogenetic response was confirmed in one patient recovering after bone marrow transplantation, and being treated with INF, and in four others who had been treated with INF for 6-24 month. A mild reaction was observed in four cases, and no cytogenetic improvement was seen in another four, while in one case there was a cytogenetic progression. In one case (the ninth patient on chart 10 b.) temporary cytogenetic improvement was observed due to Glivec treatment following INF. From all patients treated with INF we saw major cytogenetic response in six cases, and minor in ten. 18 patients

responded with permanent CHR, two with PHR. It must be noted that CHR and major cytogenetic remission developed in one patient who got bad prognosis on being diagnosed. Effectiveness of INF can show significant individual deviation: In the first patient of chart 10 a. a low-dose (2x5 ME/week) INF induced a basically complete cytogenetic remission, while in patient 5 even a daily 10 ME dose induced only a mild cytogenetic response.

FISH tests were carried out on four patients observed for leukocytosis, but Ph negative on cytogenetic tests. In one patient 16 % of the peripheral blood cells showed BCR/ABL translocation. Due to increasing leukocytosis (leukocyte count rising up to $20x10^9$ /L), anaemia and decreasing thrombocyte count BM examination was repeated: contrary to the first, almost normal panel it reflected the chronic phase of CML. The positive result of FISH test was confirmed by a PCR test too. We started an INF monotherapy. As a result of a low-dose treatment (2x3 ME, later 3x3 ME weekly) the blood panel improved, the splenomegaly decreased.

We have also investigated the presence of t(9;22), i.e. BCR/ABL translocation in seven ALL patients. In all but one patient we examined bone marrow cells. Conventional kariotype examination did not reveal chromosome anomaly in any case. The FISH test however showed BCR/ABL translocation in one patient, in a small percentage of the cells (but exceeding the error margin). This patient proved to be therapy-resistent.

We have also performed 32 hybridisations in 14 patients suffering from AML (partly with locus-specific, partly with centromere specific probes). In the first, therapy-resistant patient the test revealed monosomy of chromosome 18. In the second patient monosomy of chromosome 7 was confirmed, whereas in the next one monosomy of the chromosome 7 was only suspected. In one case (the sixth patient in chart 13.) the disease was originally CLL. Later, as a second disease, AML was confirmed, which proved to be BCR/ABL + and therapy resistant. The above abnormalities could not be detected with conventional cytogenetic tests. In the next 8 patients (chart 14.) with suspected acute promyelocytic leukaemia, FISH test was used to examine AML M3 specific chromosome aberration, the presence of t(15;17). In five out of eight patients translocation, and AML M3 was confirmed. In t(15;17) negative cases (despite a morphological resemblance to promyelocytic leukaemia) the final diagnosis was myelomonocytic leukaemia, CML blast phase, and DMPS transforming into AML. Translocation test was done partly for diagnostic reasons, partly to follow up patients: in case of the fist and fifth patient the assessment of remission was helped and complemented by negative FISH results. In this patient group it is

especially worth stressing the importance of FISH tests, as cytogenetic tests did not reveal the presence of t(15;17).

We examined 13 patients with CLL (chart 15.). We examined primarily the trisomy of the chromosome 12, which is one of the most frequent genetic anomalies in CLL. We did not find numeral disorder of the chromosome 12. Chromosome aberration was found only in one patient: earlier CGH examination showed trisomy of the chromosome 7. The conventional cytogenetic test (due to the similarity of chromosomes) suggested trisomy of the chromosome 8. By using the chromosome 7 centromere-specific probe the numeral disorder of the chromosome 7 could be clearly confirmed.

And finally the numerical anomaly (80% monosomy) of the chromosome 7 could be proved in a DMPS patient, whose disease transformed into AML.

Discussion

1. It is well known that in myeloproliferative diseases both irregular platelet counts and acquired disturbed function can coexsist, which if recognised may help set up correct diagnosis.

Various laboratory tests had been described in the literature, which may help distinguish between primary and secondary thrombocytoses; there is however no single, sufficiently reliable method, which would serve as a standard test too. Based on our observations, it can be claimed that in myeloproliferative thrombocythaemia platelet retention is decreased, the filter closure time is substantially prolonged.

The result of the filter-test proved to be positive in most patients who had been treated for chronic myeloproliferative diseases with elevated platelet count.

Our results suggest that primarily the values of the R-II samples anticoagulated with citrate can be applied as screening tests. Blocking drop count and R-I values are less sensitive.

The O'Brien filterometer used as a screening test helps differential diagnosis of the conditions with elevated platelet count. If results are normal it is more reasonable to clarify known reasons of a reactive thrombocytosis. Abnormal results (R-II) however would encourage detailed haematological checkup.

The shearing forces affecting the platelet induce GP Ib and GP IIb/IIIa expression on the platelet membrane, which in the presence of vWf results in thrombocyte-aggregation. It seems a logical conclusion that the reason for the abnormal filter-test results should be (at least partly) the inadequate connection between GP Ib and GP IIb/IIIa - vWF. It may therefore be claimed that the observed deviations in MP, the abnormal filter-test results are due to an acquired default of the platelet membrane GP IB and GP IIb/IIIa. In order to confirm this assumption, the examination of platelet membrane glycoproteins in CMPD with elevated thrombocyte count is being planned.

Future examinations are aimed at comparing diagnostic value, sensitivity and specificity of the O'Brien filter test and PFA (using parallel examinations). During patients' follow-up we are also planning to monitor treatment (hidroxiurea, interferon, anagrelid, aspirin) by repeated examinations. These tests may assist the choice of a more effective drug therapy -or combination of drugs -, and thus the optimal treatment of patients.

2. The extremely small NO production of washed human platelets could be well examined in the in vitro system, in the presence of L-arginin, with the electrochemical method that we applied.

Using the well known and frequently applied competitive inhibitors of the NO synthase the NO reacton decreased by more than 95% confirming the specificity of the results concerning NO production.

Our examinations support the fact that inactive platelets do not produce NO in in vitro circumstances.

Our data imply that the thrombocyte NO production in the in vitro system is significantly decreased in myeloproliferative thrombocytoses.

With the help of the results of the O'Brien filterometer test and the platelet NO production test we can provide a better explanation for the double-faced complications - both thrombotic and haemorrhagic - in myeloproliferative thrombocytosis: the acquired thrombocytopathy, the GP Ib, GP IIb/IIIa default would increase the tendency to haemorrhagic complications, whereas decreased NO production (inducing a more active platelet function) would rather increase the tendecy to thrombotic, vascular complications. (These data can be complemented with the results of the plasma F XIII level of our 33 patients suffering from primary thrombocytosis: the plasma F XIII level and activity was higher in myeloproliferative thrombocytosis, which again can contribute to the development of thrombotic, vascular complications.)

In concert with our assumption, and the literature it can be claimed that a “special” quantitative and qualitative platelet anomaly is present in myeloproliferative diseases.

3. We observed laboratory abnormalities implying damaged endothelium in posttransplantational diabetes mellitus, damaged fibrinolysis and increased platelet-activation. These deviations may be in a cause-effect relationship with the well known increase – 5-7 times higher than that of the healthy population - in cardiovascular morbidity and mortality.

The significantly enhanced platelet-activation can play a key role both in the progression of atherosclerosis, i.e. in the pathogenesis of the macroangiopathy, and in the development of renal lesions (microangiopathy). The latter is supported by the significant correlation between creatinine and ßTG.

Providing better lipid- and blod-sugar values we can delay angiopathy, and cardiovascular complications, as the extent of the endothelium erosion and fibrinolysis damage will be less. Literature data claim that elevated levels of triglyceride can be seen during chronic rejection. According to our examinations the elevated triglyceride levels are associated with higher PAI-I activity, that is, decreased fibrinolysis. This latter fact helps development of small-vessel thromboses, i. e. vascular rejection.

Cyclosporin A alone is known to be barely diabetogenic, administered with steroids however, it raises the incidence of diabetes. When steroid and CyA are applied in combination they intensify the potencial for diabetes, as well as damage of endothelium and fibrinolysis. The correlation between the time between initiation of transplantation and decrease of endothelium erosion and fibrinolysis supports the claim that immunosuppressive regimens containing steroids for a prolonged period are toxic, and have a salient effect on the presentation and progress of complications. Thus hypofibrinolysis can lead to deterioration of the allograft function, appearance of chronic graft-insufficiency, vascular rejection and increasing atherosclerosis.

According to some data Cyclosporin A monotherapy – steroid-free immunosuppression – can provide similar or even more persistent graft function and survival.

Modern immunonosuppressive regimens usually ward off immune rejection, but simultaneously facilitate chronic vascular rejection. More adequate immunosuppressive therapy (steroid-free protocols), and risk-adapted immunosuppression therefore should be based on haemostasis research.

4. A more precise knowledge and mapping of genetic anomalies seem more and more important in the diagnosis and treatment of acute and chronic leukaemias. In some diseases like CLL, risk-adapted treatment gets more and more emphasis, for which various factors of prognosis are indispensable. Chromosome aberrations, like numerical abnormalities or deletions, which are often revealed by cytogene tests, but in a number of cases remain hidden, have a significant importance among prognostic factors. With the help of CGH, genetic analysis of leukaemia cells comprising the whole genome –more precise definition of chromosome irregularities, deletions of a prognostic importance - becomes possible. On the basis of our few but successful tests we are of the opinion that in the future more frequent CGH tests complemented with conventional cytogenetic examinations will facilitate more precise recognition of genetic anomalies thus contributing to better, risk-adapted treatment, and discovery of further, important prognostic factors.

The quality of the cytogenetic response proved to be important, independent factor, substantially influencing survival in CML (with interferon or allogenic bone marrow transplantation). Cytogenetic response correlates with survival: a better response would imply prolonged survival. With the Glivec treatment applied lately survival is expected to become even longer, but due to the short time since its introduction data can not yet be assessed. A logical consequence of the above is the grave importance in revealing bcr/abl translocation (both to diagnose and to follow up the disease). Based on our examination the practical magnitude of FISH tests in examining bcr/abl translocation can be summarised as follows:

1. Refining diagnosis (a better assessment of Ph-positivity, differential diagnostics of leucocytoses, recognition of atypical and initial stages of CML)
2. Both bone marrow and peripheral blood samples can be examined.
3. Applicable in patients who cannot be examined with cytogenetic tests.
4. Provides more precise data on chromosome aberrations than conventional cytogenetic examinations.
5. Shows the efficiency of INF and other treatment modalities by analysing the change in the ratio of Ph + cells, which is an important feature of success (or failure). It is disputable if the FISH test is appropriate for cytogenetic follow up of patients treated with Glivec.

The data concerning this aspect are rather controversial in the literature. We are trying to clarify this point by continuing the research.

6. Assessment of indication for bone marrow transplantation.
7. Posttransplantational follow up of patients.
8. As a result of INF treatment a major cytogenetic response can develop even in groups with a bad prognosis, which is of great significance in prolonging the chronic phase, in providing better survival time.
9. As opposed to data from previous cytogenetic tests demanding metaphases, the presence of a high percentage (95-100%) of Ph-chromosome cannot necessarily be shown in untreated CML patients' bone marrow or blood samples, by using interphase cytogenetic (FISH) test either. This may affect the assessment of the presently applied criteria of residual normal haematopoesis, and the cytogenetic response.

The FISH technique, can also be well applied for AML and ALL to check the bcr/abl translocation, for revealing the diagnostic t(15;17) in AML M3, and for cytogenetic following up of patients. It may also play an important role in mapping numerical and structural chromosome anomalies in acute leukaemias. Despite the fact that among our CLL patients we only observed chromosome anomaly once (the trisomy of the chromosome 7 as a rarity), we are planning the continuation of FISH tests in CLL, expecting more precise definition of genetic abnormalities, which would influence treatment regime and prognosis.

In sum we want to apply the molecular biological tests – both the ones already in use and the planned complementary matrix-CGH tests – in order to improve treatment of patients suffering from haematological malignancy. Furthermore we will try to work out whether the recent claim that Glivec can result in genetic instability can be supported, and how this then would relate to the presence of certain unfavourable cytogenetic prognostic factors (like 9q deletion). The FISH technique can also be applied to examine genetic instability: cytogenetic deviances implying acceleration (e.g. +8, +19, +20, i(17q), double Ph chromosome) can be examined and followed up with appropriate probes (e.g. centromere-specific, locus-specific). The matrix-CGH can make the recognition of the genotype predisposing to the refraction of therapy (e.g. Glivec), as well as the tracking of clonal

evolution more effective. Besides CML, we also expect a more complete and more precise recognition of genetic prognostic factors in multiple myeloma, CLL and acute leukaemias, which then will facilitate a more sophisticated and more complex risk-adapted treatment.

Summary

1. Differentiating between thrombocytosis in myeloproliferative diseases and reactive thrombocytosis can sometimes cause serious problems in differential diagnostics. In order to differentiate between primary and secondary thrombocytosis we examined the applicability of the nationally less widely known O'Brien filter-test. Using citrate as anticoagulant the sensitivity and specificity of the filter test (R-II values) proved to be 77.1% and 94.4 % respectively. On the strength of our results the O'Brien test is useful in the differential diagnosis of conditions with elevated platelet count. In case of normal values the known causes of reactive thrombocytosis should be considered and investigated, whereas in case of abnormal values haematological examination of the patient is called for. We are also planning to use the filter test (together with PFA) in order to plan the therapy (to select the appropriate drug, or combination of drugs).

2. According to the research of recent years the NO synthase (iNOS, ecNOS) can also be found in thrombocytes, getting activated during activation and aggregation of platelets. The minute amount of NO (in the thrombocyte) produced during activation of platelets probably plays an important role in blocking further activation, adhesion and aggregation of platelets. We activated washed platelets of healthy subjects and of patients suffering from chronic myeloproliferative diseases with elevated platelet count with various agents (e.g. thrombin, collagene, epinephrine, etc.). In the patient group we could observe a significantly decreased NO-response. We assume that the reduced NO production can lead to a more active platelet-function, thus contributing to angiopathy and thrombotic complications.

3.The most important causes of morbidity and mortality after kidney transplantations are the accelerated atherosclerosis and thrombosis. Decreased fibrinolysis and lipid-deviations play an important role in their appearance. Posttransplantational PAI-1

activity increase has already been proved, but changes of other parameters of the haemostasis (e.g. tPA, TM) are only partially known, and data are often controversial.

We examined serum PAI-1, tPA, ß-TG and sTM levels postoperatively in 53 kidney transplant patients, who developed diabetes induced by immunosuppressive therapy (steroid+ cyclosporin A ± azathioprin ± mofetilium mycophenolicum). We found that PAI-1 activity, and tPA levels were more than twofold as compared to the control group indicating an increased risk of atherogen. The threefold increase in sTM suggests endothelium damage. The ß-TG level showed the most remarkable increase indicating an elevated in vivo platelet activity. In case of postoperative diabetes induced by immunosuppressive therapy the appearance of hypofibrinolysis as well as increased thrombogenity and accelerated atherosclerosis must also be considered, which may on the one hand lead to cardiovascular morbidity and mortality, and graft dysfunction and its progression on the other. The above haemostatic results support the fact that immunosuppressive therapy-induced diabetes gravely affects the vascular system, thus preference should be given to non-steroid immunosuppressive protocols.

4. In haematological malignancies defects of the genome of tumour cells are essential in the appearance, biological nature and clinical process of leukaemias. A reliable demonstration of the chromosome aberrations can therefore be essential in setting up the correct diagnosis, assessing prognosis, planning treatment and following up patients.

The main advantage of CGH is the facilitation of a comprehensive genetic examination comprising the complete genome. We hope to use this method in order to examine and acquire information about important anomalies of a prognostic and pathogenetic value and to be able to track clonal evolution much better.

The FISH technique is a useful tool in demonstrating chromosome deviations in a fast and efficient way. It is applicable for recognising chromosome-irregularities that are essential for diagnosis and prognosis, for monitoring genetic aberrations, i.e. tracking progress. As our results show, by using the FISH-technique we managed to examine the presence of BCR/ABL translocation and its quantitative change both in acute and chronic leukaemia, which is important both in setting up diagnosis, differential diagnosis, and in patients' follow-up, furthermore in assessing success of therapy. Both bone marrow and peripheral cells can be examined basically with the same success, which means that FISH-examination of peripheral blood samples is appropriate for cytogenetic follow-up of patients, there is no

need to do bone-marrow examinations on a regular basis. Similarly to BCR/ABL translocation other, structural and numerical irregularities can also be effectively examined.

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