EVALUATION OF NOVEL LABORATORY METHODS USING WHOLE BLOOD TESTING IN DIFFERENT DISTURBANCES OF HAEMOSTASIS

Ph.D. thesis

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INTRODUCTION

Haemostasis is the consequence of balanced interactions of cellular and molecular components responsible for the maintenance of an intact circulation. Dysfunction of cells or proteins participating in the haemostasis system may result in thrombophilia or a haemorrhagic diathesis. Hypercoagulable states can present themselves as arterial or venous thromboembolic disease. The major cause of death and disability in the developed world is arterial vascular disease. Thrombosis plays a central role in the pathogenesis of acute coronary syndrome (ACS). The efficacy of various antiplatelet agents in preventing cardiovascular and thrombotic complications has been established in large-scale clinical trials. However, due to the considerable individual variability in the responsiveness to various antiplatelet agents, which has been shown to have an impact on clinical outcome, there is a demand for simple assays to determine the effectivity of antiplatelet treatment. We were the first to describe a new, promising whole blood aggregometry, called multiple electrode aggregometry (MEA) and thus prepare it for further studies.

Deep vein thrombosis (DVT) and pulmonary embolism represent different manifestations of the same clinical entity, which is referred to as venous thromboembolism (VTE). The incidence rate of a first venous thrombosis is 1 to 2 events per 1000 patient-years, and about 30% of patients develop recurrence within the next 10 years. Mortality rates are high, and the long-term complications including chronic pulmonary hypertension and post-thrombotic syndrome may develop. In some cases, risk factors for both venous and arterial disease may be present, making the clinical picture more complex. Venous thromboembolism is now recognized as a complex, multifactorial disease, involving both environmental exposures (e.g. clinical risk factors) and both genetic and environmental interactions. In order to improve survival, avoid recurrence, prevent complications, and reduce health care costs, the occurrence of VTE must be reduced. To reduce VTE incidence, persons at risk for VTE must first be identified. Thrombophilia (the tendency to develop thrombosis) can be inherited, acquired, or both. There is no single haemostasis laboratory test that has the capacity to accurately demonstrate the clinical effects of procoagulant or anticoagulant interventions up to now, and is capable of diagnosing overall hypocoagulation or hypercoagulation states of blood. Thrombelastography, which is a global haemostatic method, is potentially capable of diagnosing thrombophilic states in the most natural environment: in whole blood. We will discuss the applicability of rotation thrombelastography in detection of thrombophilia in VTE patients.

AIMS

I. Aims of the study with multiple electrode aggregometry

1. Validating this novel impedance aggregometry method by using platelet agonists ADP, collagen, and thrombin receptor-activating peptide-6 (TRAP-6).

2. Examining the in vitro effect of two anticoagulants: the commonly used sodium citrate and the direct thrombin inhibitor hirudin.

3. Comparing MEA to another sensitive method for platelet aggregation: single platelet counting, using the same agonists.

4. Evaluating the in vitro platelet aggregation inhibitory effects of aspirin and the ADP scavenger enzyme apyrase as measured by MEA and SPC.

5. Investigating the contribution of the two platelet ADP receptors ($P2Y_1$ and $P2Y_{12}$) in platelet aggregation by using the $P2Y_1$ and $P2Y_{12}$ antagonists, i.e. MRS2179 and AR-C69931MX.

6. Testing MEA's ability to detect the aggregation inhibitory effect of per os clopidogrel treatment ex vivo, exhausting the synergistic effect of prostaglandin E_1 (PGE_1).

II. Aims of the examination of ROTEG® method in thrombophilia

1. Examining the applicability of rotation thrombelastography to detect hypercoagulation state in patients with venous thromboembolism, using different assays.

2. Evaluating the value of rotation thrombelastography in the diagnosis of thrombophilia.

3. Comparing the global coagulable capability of the two gender's whole blood measured by rotation thrombelastography.

4. Testing the sensitivity of two assays, INTEG and nNATEG to sodium-heparin and low molecular weight heparin.

MULTIPLE ELECTRODE AGGREGOMETRY

1. Introduction

The study of the role of platelets in the pathogenesis of ischemic vascular diseases and the monitoring of anti-platelet drug effects in patients with cardio- and cerebrovascular diseases require reliable platelet function tests. The most commonly used method is light transmission aggregometry (LTA, "Born" aggregometry) employing citrated or heparinised PRP. Disadvantages of this technique include the need of centrifugation to separate other blood cells from platelets, the loss of especially giant platelets.

For these reasons, novel methods have been introduced to measure platelet aggregation in whole blood. Single platelet counting (SPC) is one of these methods, which is characterized by the measurement of the loss of platelets after stimulation of anticoagulated blood by using conventional haematology analyzers.

Another method to measure platelet aggregation in whole blood is impedance aggregometry, which was introduced by Cardinal and Flower. It is based on the attachment of platelets on two platinum electrodes, which results in an increase of electrical resistance between the electrodes. The change of resistance is proportional to the amount of platelets sticking to the electrodes. Recently, a novel instrument to measure platelet aggregation in diluted whole blood by impedance aggregometry has been developed. This instrument uses disposable test cells with duplicate impedance sensors. The method is called multiple electrode aggregometry (MEA) because it uses 4 electrodes per test cell.

In our study we compared the novel multiple electrode aggregometry with single platelet counting measuring spontaneous platelet aggregation, ADP-, collagen-, and TRAP-6-induced platelet aggregation. We investigated the effect of two anticoagulants, and the inhibitory effect of aspirin, apyrase and the ADP receptor $P2Y_1$ receptor antagonist MRS2179 and the $P2Y_{12}$ receptor antagonist AR-C69931MX on platelet aggregation. Additionally, we tested MEA's ability to detect the aggregation inhibitory effect of per os clopidogrel treatment *ex vivo*.

2. Methods

2.1. Preparation of blood

Blood was collected from healthy volunteers into a plastic syringe containing either 1/10 volume recombinant hirudin or sodium citrate after informed consent was obtained. All volunteers denied taking any medication which might affect platelet function in the two weeks preceding the experiments. For some experiments, acetylsalicylic acid (ASA, final concentration in blood 1 mmol/l) was dissolved in the sodium citrate solution or saline solution before addition of hirudin, to achieve total dissolvement. In case of the ex vivo experiments, blood was taken from six healthy volunteers before and 3, 6 and 78 hours after administration of the first dose of clopidogrel (300 mg loading dose on day 1 and 75 mg on day 2, 3 and 4) and at least 2 weeks

after the last drug ingestion. Measurements were performed between 0.5 and maximal 4 hours after venipuncture.

2.2. Measuring platelet aggregation by multiple electrode aggregometry

Whole blood aggregation was determined using a new generation impedance aggregometer (Multiplate® analyser, Dynabyte Medical, Munich). The device has 5 channels for parallel tests, and a single use test cell with duplicate impedance sensors, each consisting of 2 straight electrode wires. The impedance change determined by each sensor is recorded independently. Due to the use of 2 duplicate electrodes per test cell the technique is called "multiple electrode aggregometry" (MEA). During the analysis the sample-reagent mixture is stirred using a discardable magnetic stirrer. In one experimental series, a special version Multiplate® apparatus with a different stirring device was tested in order to analyze the effect of turbulence on platelet aggregation. In this apparatus, the stirrer was turned alternately to the right and left by 180°. In order to attain sufficient flow in the cup a larger (6x3 mm vs. 4x2 mm) stirrer was applied.

For each measurement, 300 µl blood is required which was diluted with 300 µl saline in order to prepare a 1:1 dilution of whole blood. The appropriate dilutions of antagonists were added to the saline, before the incubation. After 3 minutes of incubation and stirring at 37°C, the measurements were started by adding 12 µl of the appropriate agonist solution or saline to the 1:1 diluted blood. The impedance change caused by the adhesion and aggregation of platelets on the electrode wires was continuously detected. The results registered by the two sensors provided two aggregation curves. The mean values of the two determinations are expressed in arbitrary "aggregation units" (AU). The test time was typically 5 minutes. The area under the aggregation curve (AUC) was used to express the aggregation response over the measured time (AU*min).

Aggregation was triggered using ADP, collagen or TRAP-6. Platelet aggregation did not alter from 30 to 240 minutes after venipuncture when measured by MEA. The method's reproducibility (intra-assay variability) was found to be 6±3% (mean coefficient of variations [%CV] ±SD, n=8). The maximal aggregation (AU) and the area under the aggregation curve (AUC) showed a linear correlation in case of ADP and TRAP-6 -induced platelet aggregation. The correlation was exponential in the case of collagen-induced platelet aggregation.

2.3. Single platelet counting

Single platelet counting (SPC) was performed as previously described by Fox et al., and Haseruck et al., with the exception that diluted blood (1:1 dilution with saline) was used in order to make the results comparable with those of the MEA measurements. Aliquots of diluted blood (15 µl) were removed from the Multiplate® test cells and transferred into a fixation buffer (30 µl). Fixed samples were counted using the Sysmex Platelet Counter PL-100 (TOA Medical Electronics, Kobe, Japan). After counting and a subtraction process, the number of the particles of the size of a single platelet is arrived at. Percentage aggregation was calculated as percentage loss of single platelets compared to baseline count. All platelet counts were done in duplicate.

2.3.6. Statistical analysis

Results are reported as mean ± SD from 4-8 experiments conducted on different blood samples. Intra-assay, intra- and interindividual variabilities were expressed as %CV (coefficient of variations, calculated as SD/mean x 100). Intra-assay variability was determined in two independent donors by assaying the same blood 4 times simultaneously. Half maximal effective concentrations (EC_{50}) were generated manually from every single dose-response curve, based on the following equation: $Y_{50}=(y_{max}-y_{min})/2+y_{min}$, the fitting X_{50} was read from the graph (where Y_{50}: half of maximal aggregation, y_{max}: maximal aggregation (AUC), y_{min}: control, without platelet stimulus AUC). Subsequently, the EC_{50}s were statistically evaluated (mean ± SD, t-tests). Half maximal inhibitory concentrations (IC_{50}) were determined correspondingly. Statistical comparisons of samples were performed by paired and unpaired Student`s t-test or linear regression analysis using Microsoft Excel 2000. Differences were considered significant when p was < 0.05.

3. Results

3.1. Spontaneous platelet aggregation and ADP-, collagen- and TRAP-6-induced platelet aggregation as measured by multiple electrode aggregometry

To evaluate the method, we measured *spontaneous platelet aggregation*, platelet aggregation induced by a „weak" platelet agonist ADP, and two „strong" platelet agonists, collagen and the PAR1 receptor agonist TRAP-6. In multiple platelet aggregometry a small, but considerable amount of spontaneous platelet aggregation was found, which ranged from 0-123 AU*min during the measurements. However, when turbulent stirring was applied (alternate movement of the stirrer to right and left by 180°, as described above) spontaneous aggregation was much higher (280±108 AU*min, mean±SD, n=6 in hirudin-anticoagulated blood) as compared to normal, circular stirring (37± 23 AU*min, mean±SD, n=8, p<0.05).

ADP induced a moderate platelet aggregation in anticoagulated whole blood in a time- and dose-dependent manner. Maximal platelet aggregation induced by ADP was highly variable between different donors, it ranged from 27 AU*min to 900 AU*min. When ADP-induced platelet aggregation was assessed for the same two donors on different days over a time period of 4 months, platelet aggregation was fairly constant. For donor "A" it ranged from 276 to 435 AU*min (365±68, mean±SD, n=5, CV: 18%), and for donor "B" it ranged from 413 to 483 AU*min (448±49, mean±SD, n=3, CV: 11%).

Collagen induced platelet aggregation obtained with impedance aggregometry started after a timelag, which changed in a dose- and donor-dependent manner. The maximal aggregation induced by collagen (2.5 µg/ml) in citrate- and hirudin-anticoagulated blood varied between different donors. It ranged from 225 to 899 AU*min. Maximal aggregation after collagen-stimulation varied less than after ADP stimulation of blood.

TRAP-6-induced platelet aggregation was characterized by a rapid initial response without a lag phase and a rapidly reached maximal aggregation. The maximal aggregation induced by TRAP-6 (20 µmol/l) in citrate- and hirudin anticoagulated blood varied between different donors

and ranged from 230 to 1028 AU*min. The interindividual variation of aggregation was less after TRAP stimulation than after stimulation with ADP.

When measured by MEA, spontaneous platelet aggregation did not correlate with stimulus-induced platelet aggregation of citrate- or hirudin- anticoagulated blood. ADP-, collagen- and TRAP-6-induced maximal platelet aggregation showed a linear correlation with each other in blood anticoagulated with citrate or hirudin.

3.2. Effect of different anticoagulation techniques

To investigate the effect of anticoagulation, we measured platelet aggregation parallelly in both citrate- and hirudin-anticoagulated whole blood. Spontaneous platelet aggregation was higher in citrated blood than in hirudin-anticoagulated blood. In hirudin-anticoagulated blood, but not in citrated blood, pre-treatment with apyrase significantly reduced spontaneous platelet aggregation. Aspirin had no effect on spontaneous platelet aggregation when either anticoagulants were used.

In the case of agonist-induced platelet aggregation we observed that all agonists induced higher aggregation in hirudin-anticoagulated blood as compared to citrated blood. Maximal platelet aggregation induced by ADP was lower in citrated blood (304 ± 136 AU*min) than in hirudin-anticoagulated blood (483 ± 224 AU*min, mean$\pm$SD; n=20; p<0.00001). This difference could be observed for all ADP concentrations used, but was not apparent when measured by single platelet counting.

The maximal aggregation induced by collagen was again lower in citrate- than in hirudin-anticoagulated blood. The values (mean$\pm$SD) were 421 ± 128 AU*min and 674 ± 110 AU*min, respectively (p<0.00001, n=19). The EC_{50} of collagen was lower in citrated than in hirudin-anticoagulated blood, indicating that the platelets were more, rather than less, sensitive to collagen-stimulation in citrate- blood as compared with hirudin-blood. TRAP-6-induced platelet aggregation was higher in hirudin-anticoagulated blood than in citrated blood measured by MEA. The mean$\pm$SD was 691 ± 184 AU*min and 531 ± 194 AU*min in hirudin- and citrate-anticoagulated blood, respectively (p<0.0001, n=15).

3.3. Comparison of multiple electrode aggregometry and single platelet counting

Spontaneous platelet aggregation during stirring of diluted whole blood was observed by both single platelet counting and MEA. By comparing the time-course of ADP-induced platelet aggregation measured by single platelet counting or MEA, it was observed that maximal platelet aggregation was reached more rapidly according to single platelet counting. Also, platelet aggregation induced by 1 µmol/l ADP was partly reversible according to single platelet counting, which was not observed with MEA. The dose-response curves for ADP were similar in both methods. However the EC_{50} of ADP measured by single platelet counting tended to be lower than when measured by MEA.

The collagen dose-response curves were different in both methods of aggregation measurement. The half maximal effective concentration (EC_{50}) of collagen to induce platelet aggregation was significantly lower when aggregation was measured by single platelet counting

7

as compared to MEA, both in citrate- and hirudin-anticoagulated blood. TRAP-6, as a strong platelet aggregation-inducing agent, caused irreversible aggregation at all examined concentrations as measured by MEA. The dose-response curves of TRAP-6 were similar in both methods of aggregation measurement shown by comparison of the EC_{50} values. No significant effect of the type of anticoagulation was found when ADP-, collagen-, or TRAP-6-induced maximal platelet aggregation was assessed by single platelet counting.

3.4. Evaluation of the in vitro platelet aggregation inhibitory effects of aspirin and apyrase

In clinical settings, the two most commonly used platelet aggregation inhibitors target the cyclooxygenase-1 (COX1) enzyme and the ADP receptor $P2Y_{12}$. We used the COX inhibitor aspirin and the ADP scavenger enzyme apyrase in vitro to test MEA's ability to detect their action in comparison with single platelet counting. As already mentioned above, spontaneous platelet aggregation was significantly reduced by pre-treatment with apyrase, but not with aspirin in hirudin-anticoagulated blood. No effect of apyrase or aspirin on spontaneous platelet aggregation measured by MEA in citrated blood or by single platelet counting in citrated or hirudin-anticoagulated blood was found.

On the other hand, when turbulent stirring was applied (alternate movement of the stirrer to right and left by 180°) the high spontaneous platelet aggregation could be reduced by aspirin and by apyrase which indicates a role of TxA_2 released from activated platelets and ADP released from activated platelets and/or damaged red blood cells.

The collagen-induced platelet aggregation measured by single platelet counting or MEA was partially inhibited by the ADP scavenger enzyme apyrase (10 U/ml) and aspirin (1 mmol/l). The inhibitory effect of aspirin was more pronounced when aggregation was measured by MEA in comparison to single platelet counting. Inhibition by apyrase was most pronounced in hirudin-anticoagulated blood when aggregation was measured by MEA.

Aspirin did not inhibit TRAP-6-induced platelet aggregation according to single platelet counting or MEA in citrate- or in hirudin-anticoagulated blood. Apyrase slightly inhibited platelet aggregation at various TRAP-6 concentrations in both single platelet counting and MEA.

3.5. Investigating the contribution of the two platelet ADP receptors ($P2Y_1$ and $P2Y_{12}$) in platelet aggregation

3.5.1. Evaluation of the effective inhibitory concentrations of the ADP receptor antagonists MRS2179 and AR-C69931MX on ADP-induced platelet aggregation

ADP-induced platelet aggregation measured by MEA was concentration dependently inhibited by the ADP receptor $P2Y_1$ inhibitor MRS2179 and the $P2Y_{12}$ inhibitor AR-C69931MX. The aggregation was completely inhibited by certain concentrations of the antagonists (>100 µmol/l MRS2179 and >100 nmol/l AR-C69931MX). We determined the half maximal effective inhibitory concentrations (IC_{50}) of platelet aggregation induced by 5 µmol/l ADP of the antagonists using different anticoagulation techniques. The IC_{50} values for AR-C69931MX were 3 nmol/l in hirudin-anticoagulated blood and 4 nmol/l in citrated blood, while for MRS2179 they

were 1.5 µmol/l in hirudin-anticoagulated blood and 6 µmol/l in citrated blood. Very low concentrations of MRS2179 (0.1 µmol/l) seemed to enhance platelet aggregation to some extent (by 20%). Based on these findings 1 µmol/l or (in case of the ex vivo experiments) 100 nmol/l concentration of AR-C69931MX and 100 µmol/l of MRS2179 were chosen in order to evaluate the role of the ADP receptors in spontaneous platelet aggregation or platelet aggregation induced by different agonists.

3.5.2. Inhibition of spontaneous platelet aggregation by MRS2179 and AR-C69931MX

The spontaneous platelet aggregation as measured by MEA was significantly inhibited by both ADP receptor antagonists in hirudin-anticoagulated blood; there was no detectable additive effect between the two antagonists when added together. Spontaneous platelet aggregation was 44.9 AU*min, 17.0 AU*min, 15.2 AU*min and 13.9 AU*min for control, with the addition of 1 µmol/l AR-C69931MX, 100 µmol/l MRS2921, or both, respectively (results are mean ± SD). These results indicate that spontaneous platelet aggregation by trace amounts of ADP in stirred hirudin-blood is mediated by activation of both the $P2Y_1$ and $P2Y_{12}$ receptor.

In citrated blood, however, only the $P2Y_1$ antagonist MRS2179 had statistically significant inhibitory effect (it reduced spontaneous platelet aggregation by 40%), indicating that only $P2Y_1$ activation is required. Spontaneous platelet aggregation was 36.2 AU*min for control, 32.4 AU*min, 21.7 AU*min and 18.9 AU*min, with the addition of 1 µmol/l AR-C69931MX, 100 µmol/l MRS2921, or both, respectively (results are mean ± SD).

3.5.3. Inhibition of collagen–induced platelet aggregation by MRS2179 and AR-C69931MX

Both ADP receptor antagonists showed approximately the same effect if hirudin anticoagulation was used. MRS2179 (100 µmol/l) inhibited platelet aggregation induced by collagen (0.5-1-2.5 µg/ml) by 71.6-49.1-39.8 (8.1-24.9-17.3) %, respectively and AR-C69931MX (1 µmol/l) by 62.0-55-30 (20.8-10.7-9.9) %, respectively (data are mean (SD)). MRS2179 had less effect in citrated blood, which difference was statistically significant, when aggregation was induced by 1 or 2.5 µg/ml collagen. The combination of both antagonists showed synergism in inhibition of collagen induced platelet aggregation.

If we relate all aggregation data to the collagen-induced platelet aggregation achieved in hirudin blood without adding an antagonist (100%), it is apparent that the relative amount of the residual platelet aggregation was about the same in citrated and hirudin-anticoagulated blood after adding an ADP receptor antagonist, and this reduction was enhanced when they were added together.

3.5.4. Inhibition of TRAP-6-induced platelet aggregation by MRS2179 and AR-C69931MX

There was a clear inhibition of AR-C69931MX in both anticoagulants used on TRAP-6-induced platelet aggregation, to about 25-80% of control aggregation. The higher the TRAP-6 concentrations, the less inhibition of aggregation could be achieved with AR-C69931MX. MRS2179, however did not inhibit the TRAP-6-induced platelet aggregation in citrated blood, on

the other hand, it significantly enhanced the aggregation induced by 10 μmol/l TRAP-6. In hirudin-anticoagulated blood however, MRS2179 was able to inhibit 3.75 μmol/l TRAP-6-induced platelet aggregation to the same extent as AR-C69931MX, but this inhibitory potential declined with the increase in the TRAP-6 concentration. Despite of the loss of the inhibitory effect of MRS2179, a synergism between the antagonists could be observed even at higher TRAP-6 concentrations.

3.6. Clopidogrel responsiveness measured by MEA in blood (ex vivo studies)

Control ("spontaneous") platelet aggregation seemed not to be affected by clopidogrel intake. Inhibition of ADP (5 μmol/l)-induced platelet aggregation 3h to 3 days after clopidogrel intake was dependent on the volunteer tested. In hirudin-anticoagulated blood, 2 persons (P1 and P2) showed more than 90% inhibition of aggregation (clopidogrel responsive), whereas 2 persons (P3, P5) were clopidogrel-resistant (<10% inhibition). Two persons (P4, P6) were partially responsive to clopidogrel (49 and 57 % inhibition). The maximal effect could be seen already 3 hours after the administration of the loading dose (300 mg) of clopidogrel, and the effect was fairly constant throughout the experiment. Addition of AR-C69931MX (100 nmol/l) to the test cells in vitro maximally inhibited ADP-induced platelet aggregation before and after clopidogrel-intake in all volunteers, thereby indicating the functional $P2Y_{12}$ receptor reserve after clopidogrel-intake (i.e. clopidogrel could not inhibit platelet aggregation completely). Similar observations were made, when citrate was used as anticoagulant. However, ADP-induced aggregation was again lower in citrate- than in hirudin-anticoagulated blood, and the inhibition by clopidogrel was therefore less detectable. Addition of PGE_1 (10 nmol/l in case of hirudin anticoagulated blood, 6 nmol/l in case of citrated blood) itself inhibited platelet aggregation by 30-50%, and enhanced the clopidogrel induced platelet inhibition from ~40% to ~60-85% in hirudin blood. Except of P1 and P2, whose ADP-induced platelet aggregation was completely inhibited by clopidogrel, the relative inhibition was higher when using additional PGE_1 than ADP alone. As expected from the results of incomplete inhibition of TRAP- and collagen-induced platelet aggregation by AR-C69931MX, TRAP-6 and collagen-induced aggregation was only slightly affected after clopidogrel intake. Collagen-induced platelet aggregation remained practically unchanged after clopidogrel intake. Two weeks after the last clopidogrel intake, aggregation values returned to the previous levels.

4. Discussion

We were the first to introduce a new technique for whole blood aggregometry based on the impedance method. Because of the application of a total of 4 electrodes for the analysis, the method is called "multiple electrode aggregometry" (MEA). By the application of an electrical signal, the measurement is independent of optical variables in the sample (e.g. lipemia). Each analysis provides a kinetic signal which is characterized by the area under the curve (AUC, expressed as AU*min). We found that the instrument was easy to handle and the results were highly reproducible.

We observed a great interindividual variability in both spontaneous and agonist-induced platelet aggregation when measured by MEA, and the variability of platelet aggregation was most

pronounced after stimulation with ADP. A significant correlation was shown between the aggregations induced by different agonists. No correlation was observed, however, between spontaneous platelet aggregation and stimulated platelet aggregation.

All of the investigated agonists elicited significantly higher platelet aggregation responses measured by MEA in hirudin-anticoagulated blood than in citrated blood, confirming previous findings of Wallén et al. Interestingly, spontaneous platelet aggregation according to MEA was less in hirudin-blood than in citrated blood (7% vs. 16% of maximal aggregation, respectively), which excludes the possibility of an artefact caused by hirudin. However, since spontaneous aggregation was not inhibited in citrated blood by apyrase or by aspirin, consequently neither ADP nor TXA_2 contributed to the more pronounced spontaneous aggregation. The higher spontaneous aggregation in citrate-anticoagulated vs. hirudin-anticoagulated blood could be explained by small traces of active thrombin possibly present in blood anticoagulated with citrate.

MEA was compared to single platelet counting, one of the standard techniques of platelet aggregation analysis in whole blood. We found that the results obtained by MEA compared, in general, well with the single platelet counting method in diluted blood. In hirudin-blood spontaneous platelet aggregation was significantly inhibited by apyrase when measured by MEA, which suggests a role of ADP that could be released either from red blood cells or platelets. We found that the use of a different MEA instrument causing more turbulence due to uneven stirring resulted in significantly higher spontaneous platelet aggregation. This can be explained by both the release of ADP and the formation of TxA_2 from activated platelets, since under these conditions spontaneous aggregation could be inhibited by both apyrase and aspirin.

Besides the good agreement of platelet aggregation responses determined by SPC and MEA, differences between the two methods were also observed. Single platelet counting was more sensitive in the detection of platelet aggregation induced by low concentrations of ADP (non-significant) and collagen (significant), while there was no difference in sensitivity towards TRAP-6-induced platelet aggregation. These differences are probably due to the fact that single platelet counting method measures recruitment of platelets into platelet aggregates which can be as small as two platelets, while impedance aggregometry is dependent on firm attachment of platelets onto the metal sensors. Accordingly, platelet aggregation measured by the single platelet counting method was more rapid than when measured by MEA. However, the normal blood cation concentration seems to be important for platelet spreading and platelet aggregation as measured by impedance aggregometry, since the ADP-, collagen- and TRAP-induced platelet aggregation was lower in citrate-anticoagulated blood when measured by MEA, but it did not affect platelet aggregation measured by SPC.

The sensitivity of impedance aggregometry towards low dosages of aspirin has previously been shown by Sathiropas et al. in ex vivo experiments. Riess et al. showed that the anti-platelet effect of a single oral dose of aspirin on collagen-induced platelet aggregation could be detected for a longer period by impedance aggregometry than by the turbidimetric method. In the MEA device, aspirin had a significant inhibitory effect even at the highest concentration of collagen used (2.5 µg/ml) in contrast to inhibition measured by single platelet counting.

When comparing platelet inhibition of the ADP scavenger apyrase in citrate- and hirudin-anticoagulated blood, we found that inhibition - as measured by both methods - was more pronounced in hirudin-anticoagulated blood. We also investigated the role of the two platelet ADP

receptors, $P2Y_1$ and $P2Y_{12}$ in the contribution of the effect of released ADP. When determining the effective concentrations of the ADP receptor antagonists on ADP induced platelet aggregation by MEA, regardless of the anticoagulation used, we observed, that at a certain antagonist concentration the aggregation was completely inhibited. These findings suggest that activation of both receptors is essential to the firm attachment of platelets to the MEA electrodes, and perhaps therefore to other non-physiological surfaces such as atherosclerotic plaques.

The inhibitory effect of the used antagonists was concentration dependent, in accordance with findings observed by turbidimetry or VASP phosphorilation, single platelet counting, PAC-1 expression assessed by whole blood flow cytometry. IC_{50} values of AR-C69931MX were similar in hirudin- and citrate-anticoagulated blood, whereas MRS2179 was more effective in hirudin-anticoagulated blood. This finding suggests that in citrated blood, the $P2Y_1$ receptor or its transduction pathways are already impaired probably due to the low cation concentration.

Both ADP receptor antagonists inhibited collagen induced platelet aggregation, suggesting that the secondarily released ADP is important in collagen induced platelet aggregation. We observed that MRS2179 had a markedly lesser effect in citrated blood than in hirudin blood, but the overall residual platelet aggregation was about the same in both anticoagulants. On the other hand, the platelet aggregation obtained in citrated blood was decreased related to the aggregation obtained in hirudin-anticoagulated blood. These findings suggest that this decrease in aggregation is due to the impairment of a $P2Y_1$ dependent pathway because of the not physiological divalent cation concentrations in citrated blood. As this effect could not be detected in citrated blood before by single platelet counting (measuring microaggregation), we suppose, that the $P2Y_1$ receptor dependent pathway is responsible for macroaggregate formation.

TRAP-6 is a synthetic peptide ligand of the thrombin-receptor PAR-1, a member of the G-protein coupled receptors found on platelets. We found, that TRAP-6 induced platelet aggregation can be partially inhibited by $P2Y_{12}$ receptor blockade; however, the $P2Y_1$ inhibitor MRS2179 has little effect on platelet aggregation. These findings are consistant with the fact, that activated PAR-1 directly activates $G\alpha_q$ protein, and secondarily enhances the release reaction. The released ADP promotes the platelet aggregation mainly due to the $P2Y_{12}$ receptor, since the $P2Y_1$ receptor-linked $G\alpha_q$ is already activated.

After we had demonstrated, that MEA had the ability to detect the inhibition factor of ADP receptors, we started to investigate the ex vivo platelet aggregation inhibitory effect of clopidogrel, which, along with aspirin, is one of the most widely used ADP receptor antagonists in the secondary prevention of ACS. Through its active metabolites formed in the liver, it irreversibly inhibits the platelet ADP receptor $P2Y_{12}$, resulting in decreased platelet aggregation. In an experiment on a small population of healthy individuals we demonstrated, that when measured by MEA, the currently recommended clopidogrel dose (saturation dose followed by a sustaining dose) significantly decreased the ADP induced platelet aggregation as early as three hours after the drug intake. A large variation in the extent of decrease could be observed among the volunteers. Amongst our test group 2/6 persons showed clopidogrel resistance and 4/6 persons had clopidogrel responsiveness when we compared inhibition to the platelet aggregation measured before clopidogrel intake. As we concluded, clopidogrel does not develop a full inhibition of the platelet ADP receptor $P2Y_{12}$, since in vitro added AR-C69931MX could increase the inhibitory effect. It has been previously shown, that the effects of $P2Y_{12}$ antagonists are enhanced by natural

prostaglandins such as PGE_1, as prostaglandins increase the intracellular cAMP concentrations and PKA activation, thus interfering with $P2Y_{12}$ receptor activation lowering the cAMP concentration in activated platelets. It has also been shown, that VASP phosphorilation was more effective in detecting the clopidogrel effect, if ADP was combined with PGE_1 or iloprost. In our study, PGE_1 generally enhanced the inhibitory effect of clopidogrel. In short, ADP induced platelet aggregation of hirudin-anticoagulated blood together with PGE_1 proved to be the best combination when using multiple electrode aggregometry to detect the clopidogrel effect in healthy volunteers. In our study, the maximal platelet inhibitory effect of clopidogrel was already detectable after 3 hours of drug ingestion, which is in accordance with the result of previous studies. To define the limit between responders and non-responders, larger studies would be required both in healthy populations and among ACS patients.

In conclusion, by using different agonists we demonstrated that MEA is a fast, convenient platelet function testing method that enables the measurement of platelet aggregation in diluted whole blood even in point-of-care settings. Using hirudin for blood anticoagulation proved to be better than citrate. Aggregation measurements were convergent to those obtained by single platelet counting. MEA was able to detect the inhibitory effect of aspirin, apyrase and ADP receptor inhibitors in vitro and also ex vivo at different concentration of agonists, and thus is a good candidate for testing even the early effects of antiplatelet drugs in various clinical settings.

THE USE OF ROTATION THROMBELASTOGRAPHY IN THE DIAGNOSIS OF THROMBOPHILIA

1. Introduction

Citrated plasma has been used predominantly in routine coagulation analysis, for several decades. While plasma contains the majority of the coagulation factors implicated in the coagulation process, whole blood includes phospholipid bearing cells, platelets and leukocytes with an important ability to support coagulation and fibrinolysis. Thus, choosing whole blood for coagulation analysis appears more favourable compared to study of plasma coagulation by PT- and APTT – based methods that include excessive activation. Since 95% of the coagulation takes place after the initiation of clot formation, continuous profiles of coagulation may provide additional information on the entire haemostatic process. In 1948, Hartert introduced the trombelastographic principle (TEG) that records viscoelastical changes during coagulation. The continuous coagulation profiles of thrombelastography may provide a more accurate reflection of in vivo biology, covering initiation, development and final clot strength during whole blood clot formation. Today, thrombelastography is utilized in various clinical settings, e.g. monitoring liver transplantation and cardiac surgery, it has also been used to investigate hypocoagulation, hypercoagulation and the ex vivo testing of haemostatic interventions. Although the TEGs ability to detect hypercoagulability of whole blood has been proved early and extensively in various situations, there are only sporadic reports about the detectability of thrombophilia by the method. Recently a newly developed portable TEG instrument (ROTEG® Coagulation Analyzer, Pentapharm Co., Munich, Germany) has been marketed. In this TEG, data are continuous, digital, and retrievable for further calculations. It proved to be a convenient method for overall patient-near haemostatic profiling. In our study we investigated 55 patients with clinically opposed thrombophilia and compared their ROTEG® parameters to those of 66 healthy volunteers. We examined the effect of genders on coagulation and the sensitivity of the different ROTEG® tests to heparin.

2. Methods

2.1. Subjects and preparation of blood and plasma samples

Blood was collected from 66 healthy volunteers (age: 34.9±10.7 years; male:female=32:34) and 55 consecutive patients (age: 37.6+12.6 years; male:female=12:43) with personal history of venous thromboembolic disease referred to our Haemostasis Outpatient after informed consent was obtained. All healthy subjects denied taking any medication that might affect platelet function or coagulation, in the two weeks preceding the experiments. Patients with thromboembolic disease on oral anticoagulant therapy were converted to a prophylactic dose LMWH (typically 0.01 ml nadroparine/kg or 1 mg enoxaparine/kg once daily) at least 14 days before venipuncture. Blood was only taken at least 3 months after the last VTE event, after 12 h fasting, in morning hours, 24 hours after the last dose of LMWH. ROTEG® measurements, routine haemostatic tests (PT, aPTT, fibrinogen, TT) were performed typically between 0.5-4 hours after venipuncture, except for the native

blood samples which were tested immediately after venipuncture. Plasma samples for specific factor determinations were centrifuged at 2000 g for 20 minutes, aliquoted and kept frozen at -70 C° until the tests were performed.

2.2. Screening haemostasis tests and detection of inherited and acquired thrombophilia

We determined prothrombin time, fibrinogen, activated partial thromboplastin time, thrombin time, antithrombin activity and antigen, protein C activity and antigen, protein S activity and free antigen, APC ratio, lupus anticoagulant, plasma homocysteine level, FV:Q506 mutation and FII G20210A mutation.

2.3. Rotation thrombelastography

We analyzed the blood samples by using modified thrombelastograph® coagulation analysis (ROTEG®; Pentapharm Co., Munich, Germany), which is based on the thrombelastograph® system (TEG®) after Hartert. TEG measures shear elastic modulus during clot formation and subsequent fibrinolysis. ROTEG® shows good correlation with conventional TEG® determination, and an excellent reproducibility and precision. TEG measurements produced accurate and reproducible results within 30 min up to 4 hours. ROTEG® uses a ball-bearing system for power transduction, which makes it less susceptible to mechanical stress, movement, and vibration. Furthermore, the activation of the test samples accelerates the measurement process and seems to enhance reproducibility when compared with conventional TEG analysis. The main variables of ROTEG® analysis are "clotting time" (CT), "clot formation time" (CFT), "maximum clot firmness" (MCF), the alpha-angle and the maximum lysis (ML).

The following tests were performed:

1. Native TEG (nNATEG): native whole blood was placed into the test cell immediately after venipuncture and the test was started without delay.

2. Non-activated TEG (NATEG): Just before running the assay, citrated blood samples were recalcified with 20 µL of CaCl2 0.2 M (Start-TEG; Pentapharm Co., Munich, Germany) and the test was started without an added activator.

3. Intrinsic TEG (INTEG): NATEG + activation using surface activator (partial thromboplastin from rabbit brain, 20 µL of In-TEG reagent, Pentapharm Co., Munich, Germany) for monitoring the intrinsic system (factors XII, XI, IX, VIII, X, II, I and platelets).

4. Extrinsic TEG (EXTEG): NATEG + activation using tissue thromboplastin (rabbit brain extract; 20 µL of Ex-TEG reagent, Pentapharm Co., Munich, Germany) for monitoring the extrinsic system (factors VII, X, V, II, I and platelets).

In some cases, 16 µL of appropriate dilutions of nadroparine or sodium-heparine were added to the test cells before starting the measurement.

2.4. Statistical analysis

Results are reported as mean ± SD from the measurements conducted on different subgroup of patients or healthy volunteers. Statistical comparisons of samples were performed by paired and

unpaired Student`s t-test or linear regression analysis using Microsoft Excel 2000. Differences were considered significant when p was < 0.05.

3. Results

3.1. Comparison of patients with a history of thromboembolic disease and control subjects

55 consecutive patients with personal history of venous thromboembolic disease referred to our Haemostasis Outpatient Clinic, were tested. In the group of patients with thromboembolic anamnesis the CT in INTEG assay, the CFT in INTEG and EXTEG assays were significantly shorter, the MCF in INTEG and EXTEG assays and the alpha angle in INTEG and EXTEG assays were significantly higher when compared to the control group. There were no significant differences between the two groups in NATEG assay. Surprisingly, when using nNATEG assay, CFT proved to be significantly longer and MCF and alpha angle significantly lower compared to the healthy volunteers.

3.2. Comparison of patients with positive and negative thrombophilia results

Out of the examined 55 patients 34 (61%) proved to be thrombophilia (ThP) negative, 21 (39%) were ThP positive (13 FV Leiden heterozygotes, 2 FV Leiden homozygotes, 2 FII20210 heterozygotes, 1 lupus anticoagulant positive, 1 protein C deficient, 2 hyperhomocysteinaemia patients). When we compared the results of ThP negative and positive patients, we found no significant differences; although the CT and CFT values were still significantly lower if compared to the controls in case of the INTEG assay.

3.3. Effect of low molecular weight heparin on blood coagulation measured by ROTEG®

Regarding the confusing results in nNATEG test, we carried out some additional experiments. As most of the VTE patients received LMWH prophylaxis during this period, we hypothesized a disturbing effect of LMW-heparin to the results. We tested the sensitivity of two ROTEG® assays (INTEG and nNATEG) to nadroparine, a commonly used low molecular weight heparin in vitro. We found, that nadroparine impairs whole blood coagulation in a dose dependent manner. The assay nNATEG was much more sensitive to low doses of nadroparine than INTEG (IC_{50}s for clotting time were 0.65 and 15 U/ml, respectively).

3.4. Effect of gender on blood coagulation measured by ROTEG®

Observing that the male to female ratio was significantly different between patients and controls, we investigated, if there was a difference between the genders in the control group. The results showed that there were no significant differences in nNATEG, NATEG and INTEG parameters between male and female controls. In EXTEG, however, CT and CFT were significantly shorter; MCF and alpha-angle were higher in women compared to men.

4. Discussion

We compared 66 healthy volunteers with 55 consecutive patients with personal history of venous thromboembolic disease referred to our Haemostasis Outpatient Clinic using the standard assays of the recently developed rotation thrombelastography. We demonstrated, that patients with previous thromboembolic disease were more hypercoagulable when compared to controls, using either by tissue factor or by contact activator activated assays. Previously TEG has shown a promising ability to detect known or established hypercoagulable states during pregnancy and postpartum, during the presence of cancer and in association with surgery. Although the high sensitivity of TEG to hypercoagulability has been described in numerous studies, clear association between TEG parameters and thrombophilia has not been established. O'Donnel et al. examined thrombophilia patients, and concluded, that TEG cannot be used as a sole initial screening test in patients referred for thrombophilia testing, as it fails to identify 43% of underlying thrombophilic traits.

Since the clinical parameters of patients and controls differed significantly in the male to female ratio, and previously a significant difference between ROTEG® profiles of genders was observed, we compared data between males and females. Females proved to be more hypercoagulable than males in case of tissue factor activated ROTEG®. Interestingly, Sorensen et al. described that in TEGs activated by minute amounts of tissue factor, females expressed a higher clot formation velocity as compared to males, which is consistent with our findings. On the other hand, the other assays (i.e. NATEG, INTEG, nNATEG) were not affected by genders. So despite the imbalance between the genders in our study, we can conclude that the results of the contact activator activated thrombelastography seem to be reasonable.

In the nNATEG test, oddly, the VTE patients proved to be hypocoagulable when compared to the control group. As most of the patients received LMWH as VTE prophylaxis during the investigation, we suspected that the heparin had a role in this effect. The high sensitivity of nNATEG (it was almost 100 times more sensitive than INTEG) of both low molecular weight and unfractionated heparin could explain the apparent discrepancy of the results. Activated assays can eliminate the disturbing effect of heparin traces.

Surprisingly, we did not find significant differences between the parameters of patients with thrombophilia proven by laboratory tests and those with no thrombophilia trait, although most of the found defects are not considered as severe thrombophilia. This and the relatively small case number may mask the differences detectable by ROTEG®. However, since the tested patients were clinically considered to have thrombophilia, the lack of difference between the two groups can also be explained by the existence of not yet identified prothrombotic abnormalities, which hold the same thrombotic event potential as the known ones. Although the differences between the thrombosis and the control group are small, and thus the assay is per se not capable of identifying patients with thrombotic tendency, there is a possibility of developing more sensitive assays based on rotation thrombelastography.

In conclusion, our study indicated that rotation thrombelastography, first of all INTEG assay was able to detect hypercoagulability in patients with venous thromboembolism, which was independent of the presence of laboratorially detected thrombophilic traits.

SUMMARY

New results achieved by multiple electrode aggregometry

1. We were the first to describe multiple electrode aggregometry, a novel platelet aggregation method, which proved to be a reliable and reproducible method. Different platelet agonists induced platelet aggregation in donor-, time-, and dose dependent manner, which was not affected by platelet count within the normal range.

2. We showed that when measured by MEA, as citrate seems to inhibit platelet aggregation, the use of the direct thrombin inhibitor lepirudin as an anticoagulant is preferable to the use of trinatrium citrate.

3. Comparing MEA to SPC we found, that results are highly comparable, while SPC was much more time consuming than MEA.

4. We concluded that MEA is able to detect the expected inhibitory effect of aspirin and apyrase to a greater extent than SPC.

5. MEA could detect the inhibitory effect of selective ADP receptor antagonists in vitro.

6. MEA was also capable of the ex vivo testing of clopidogrel effectivity. MEA may be a good candidate for further studies in large populations of cardiovascular patients.

New results achieved by rotation thrombelastography

1. Rotation thrombelastography was proved to detect thrombotic tendency in patient with former thromboembolism.

2. We were the first to investigate the applicability of the most commonly used ROTEG$^{®}$ tests (nNATEG, NATEG, INTEG, EXTEG) in thrombophilia, and found that INTEG especially has the ability to detect hypercoagulable state.

3. There was no difference between the ROTEG$^{®}$ parameters of TE patients with or without thrombophilic trait. This suggests that clinically both groups of patients had the same tendency to thrombosis, probably due to an underlying, so far unknown factor.

4. In the group of healthy volunteers the women seemed to have a higher tendency to thromboembolism, when compared to the men, when we used the tissue factor activated test.

5. Sodium-heparin and LMWH both inhibited the coagulation when measured by native or contact factor activated assay, the former being much more sensitive to both kinds of heparins.

In conclusion, we applied two novel whole blood haemostasis methods to measure platelet aggregation and global coagulation tendency. According to our results, both methods proved to be helpful in haemostasis research, and can also be useful in diagnosing haemostasis abnormalities.

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