THESES OF Ph.D.

The changes of leukocyte-platelet mixed aggregates formation, platelet EDRF/NO production, and haemorheology in diabetes mellitus, their role in the pathogenesis of diabetic angiopathy

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INTRODUCTION

Diabetes mellitus is a continuously growing problem all over the world. The incidence of the disease is becoming higher every year and it is estimated by the WHO that the number of diabetic patients will nearly be doubled by the year 2020.

Not only the treatment of diabetes, but also the treatment of its complications mean a great burden on society. One of the main problems is that the diabetic complications may be treated, but not cured. This is the reason why their prevention is necessary.

DCCT and UKPDS studies have demonstrated that normalisation of serum blood glucose level and blood pressure contribute largly to the prevention of diabetic complications. In this way the occurence of microvascular comlications may be reduced by nearly 40-70%. In order to more effectively decrease the incidence of vascular complications one needs to discover its pathogenesis in details. According to some new information there are certain non-glycemic factors contributing to the development of micro- and macrovascular complications like haemochromatosis heterozygosity – iron level of the body and haptoglobin polymorphysm. These factors may even be useful to forecast the susceptibility to angiopathy.

It was proven in the last years that there are a lot of factors having a role in the pathogenesis of diabetic vascular disease, faktors like enhanced oxidative stress, platelet activation, endothelial dysfunction, disturbances in haemorheology etc. Oxidative stress and postprandial hyperglycemia lasting only for a short period seem to have a major role in this respect.

Patients suffering from myeloproliferative disorders with high platelet count frequently have symptomes due to disturbances in microvascular circulation. These patients often have thromboembolic events and as a special charactaristic of these diseases they may produce bleedig complications.

This Ph.D. work is mainly written about the pathogenesis of diabetic vascular complications and to a lesser extent and only partially of the vascular symptomes of patients with different myeloproliferative disorders with high platelet counts. The main part of this work is about the formation of heterophilic leukocyte-platelet mixed aggregates in diabetic patients and in patients with myeloproliferative disorders. The association of formation of heterophilic aggregates with postprandial serum glucose elevation was also observed. The other very important part of this study is the investigation of platelet EDRF/NO production to different natural stimuli. Changes in haemorheology parameters (relative cell transit time, plasma viscosity, fibrinogen level), oxidative stress (in red blood cells as well as plasma) were measured. Hyperhomocysteinaemia and Helicobacter positivity was also evaluated.

AIMS

The aim of this study was to determine:

- If the number of heterophilic aggregates was elevated in patients with type 1 and type 2 diabetes mellitus?
- If there was an association between the formation of heterophilic aggregates and the carbohydrate metabolism (HgbA1c level)?

- If the number of heterophilic aggregates was elevated in patients with different diabetic vascular complications and if there was any association between heterophilic aggregate formation and the development of diabetic angiopathy? Had the postprandial serum glucose elevation have any effect on the heterophilic aggregate formation?
- If heterophilic aggregates had any role in the development of vascular symptomes observed in patients suffering from myeloproliferative disorders with high platelet count?
- If there was a change in EDRF/NO production of platelets from diabetic patients to different physiologic stimuli?
- What kind of changes could be obeserved in haemorheology parameters (plasma viscosity, deformability of red blood cells)?
- What kind of changes could be detected regarding to oxidative stress (in the plasma and in red blood cells)?
- Was there any kind of association between the above mentioned factors?
- Were the homocystein levels elevated in diabetic patients and had these elevated levels have any effect on the developmnet of vascular complications?
- Had Helicobacter pylori infection have a role in the pathogenesis of diabetic complications?

MATERIALS AND METHODS

The *number of heterophilic aggregates* was measured in 90 diabetic patients. Sixty-one patients with Type 2 and twenty-nine with Type 1 diabetes mellitus were investigated. The control group consisted of 23 apparently healthy persons (Table 1).

	Number of patients(n) Gender	Age ± SD (year)	Duration of diabetes ± SD (year)
Type 1 diabetes mellitus	n=29 male: 12 female: 17	36 ± 14	9,6 ± 11,7
Type 2 diabetes mellitus	n=61 male: 37 female: 24	58 ± 11	8,8 ± 5,9
Control	n=23 male: 12 female: 11	35 ± 15	-

Table 1.

None of the patients or controls received antiplatelet agents at least ten days prior to blood sampling. The method for investigating heterophilic aggregates was described earlier. Venous blood samples were briefly anticoagulated with heparin. To standardize the measurement conditions and to minimalize the in vitro platelet activation 50 µl of whole blood sample (instead of platelet rich plasma) was labelled exactly 60 minutes after blood collection by saturating concentrations of FITC-labelled CD42a and CD45 PE-Cy5 (Becton Dickinson Biosciences). After a 15 minute incubation in the dark red cells were lysed by FACS lysing solution, then samples were washed twice in phosphate buffered saline and fixed

in 1% paraformaldehyde. Flow-cytometric analysis was performed by a FacScan flow cytometer using CellQuest software by gating on CD45 positive events to exclude non-leukocyte particles. Leukocyte subsets were identified on the FL3 (CD45)-side scatter plots. Acquisition was terminated when data on 2000 monocytes was collected. Fluorescence data of individual gates was analysed on histogram plots and CD42a-FITC staining was compared to a FITC labelled isotype control where the marker was set to provide 1% positive cells. Positivity for CD42a staining was registered as percentage of the respective leukocyte subset.

In addition, investigation of the effect of postprandial serum glucose elevation on heterophilic aggregate formation has also been performed. Thirteen patients with Type 2 diabetes had the same, standard breakfast containing 50 g carbohydrate without acarbose and with 100 mg acarbose prior to the meal on the consecutive day. Blood samples were obtained before and 60 and 120 minutes after breakfast. These patients were treated by diet alone or with acarbose, three patients took sulfonylurea agents in low dose, but this drug was withdrawn during the test period. None of the patients had any kind of overt diabetic vascular complication. The standardized meal was given in the morning between 8.00 and 9.00 AM after a twelve hour fasting period.

The *EDRF/NO production of platelets* was measured as follows: washed, isolated platelets, gained from healthy persons and Type 1. and 2. diabetics, were exposed to common and physiologically relevant activators (i.e. thrombin, ADP, epinephrine, collagen, ristomycin.), and a specific electrochemical device developed for this purpose estimated NO production. NO production was also measured in the presence and in the abscence of L-NAME and L-NNA as well. 19 diabetic patients were involved in this study (7 type 1 - mean age 22,3±4,1 year and 12 type 2 patients - mean age 62,3 ± 11,4 year) and there were 13 controll subjects (mean age 38,5 ± 6,2 year).

The *EDRF/NO production of endothelium* was calculated from NO2/NO3 content of urine.

Haemorheology parameters were detected in 54 diabetic patients (27 type 1 – mean age 36 ± 14 year and 27 type 2 patients 56 ± 10 year). Red blood cell deformability - the relative cell transit time, the fibrinogen level and plasma viscosity was measured.

TAS (total antioxidant status) was measured in the plasma and it was the “inverse picture” of *oxidative stress*. To determine the oxidative status in red blood cells malondialdehyd level was detected and the result was given as nmol MDA/g Hgb.

Homocystein level was detected in 79 diabetics (15 type 1 and 64 type 2 patients) with HPLC.

Helicobacter pylori was tested in 100 diabetics (mean age 58,5 ± 12,9 year, 10 type 1 and 90 type 2 patients). UBT was carried out.

RESULTS

The results showed a significant difference in the number of monocyte-platelet heterophilic aggregates between both diabetic groups and the control group (Type 1 diabetes: 43,0±17,8; Type 2 diabetes: 34,9±12,5; control: 24,6±8,2; Type 1 diabetes vs control: $p<0,001$; Type 2 diabetes vs control: $p<0,05$; Type 1 diabetes vs Type 2 diabetes $p>0,05$). The numbers of granulocyte-platelet and lymphocyte-platelet mixed aggregates were, however, only slightly elevated and were not significantly different from the controls. There was a positive correlation between monocyte-platelet and granulocyte-platelet heterophilic

aggregates (r = 0,50 and r = 0,54). The highest number of monocyte-platelet heterophilic aggregates was found in patients with proliferative retinopathy and nephropathy. The HgbA1c level was significantly higher in the diabetic group (Type 1 diabetes: 9,11±2,60; Type 2 diabetes: 9,32±2,39; control group:5,16±0,21) but, no association was found with the number of heterophilic aggregates, and the number of aggregates also seemed to be independent of the duration of disease. There were two patients, however, in whom we have found higher circulating heterophilic aggregate number without vascular complications and in two others low aggregate number with microvascular complications (neuropathy and preproliferative retinopathy).

In Type 2 diabetes a remarkable tendency in the postprandial serum glucose elevation and the increase of monocyte-platelet aggregate formation has been found. Both serum glucose level and the percentage of monocyte-platelet aggregates elevated postprandially, but the difference was not significant in either case. Acarbose treatment diminished posprandial serum glucose elevation at already 60 minutes and ratio of monocyte-platelet heterophilic aggregates was also lower at 120 minutes compared to untreated patients.

In vitro platelet NO responses were reduced in each of the diabetic subjects, the speed of NO generation was also slow with some of the stimulants. Results can be seen in Table 2.

Table 2. The amount of NO produced by platelets (nmol/l/100000 platelets) and the speed (Km) in healthy subjects and diabetic patients.

Group	**Collagen**		**ADP**		**Adrenalin**		**Thrombin**		**Ristomycin**	
	Nano mol/l	**Km**	**Nano mol/l**	**Km**	**Nano mol/l**	**Km**	**Nano mol/l**	**Km**	**Nano mol/l**	**Km**
Control	901.3 ± 381,1	1.75 ± 1.48	377,3 ± 159,1	2,51 ± 1,98	950,2 ± 314,2	1,61 ± 1,51	1049 ± 402,2	1,66 ± 1,42	682,2 ± 292,4	1,44 ± 0,96
Type 1. Diabetes	529,1 ± 216,2	1,77 ± 1,36	188,6 ± 99,4	2,25 ± 1,23	821,9 ± 297,3	2,18 ± 0,91	555,4 ± 212,3	1,18 ± 0,50	823,0 ± 271,4	1,70 ± 1,16
Type 2. Diabetes	606,6 ± 346,5	1,31 ± 1,55	221,6 ± 178,2	1,44 ± 0,19	807,5 ± 394,5	3,87 ± 1,62	605,5 ± 286,1	1,87 ± 0,94	868,7 ± 375,0	2,37 ± 0,96

There was a significant decrease in *red blood cell de*formability (increase in relative cell transit time) in both groups of diabetic patients (Type 1: 7,36±0,81 vs. 6,03±0,30, p<0,001; Type 2: 7,00±0,79 vs. 6,03±0,30, p<0,01) that contribute to the demage of microcirculation.

Oxidative stress was increased in both the plasma and in the red blood cells in Type 1 and Type 2 diabetic patients (TAS (mmol/l): Type 1: 0,74±0,20 vs. control: 1,22±0,28, p<0,001; Type 2 diabetics: 0,83±0,27 vs. control: 1,22±0,28, p<0,05; oxidative stress in red blood cells was given as nmol MDA/g Hgb: Type 1 diab. mell.: 3,38±1,91 vs. control: 1,87± 1,22, p<0,05; Type 2 diab. mell.: 3,98±1,66 vs control: 1,87±1,22, p<0,01). Suprisingly, there was no assotiation found between decreased red blood cell deformability and increased oxidative stress.

There was no significant difference found between diabetic and control group in *plasma viscosity*, whereas the *fibrinogen level* was nearly significantly elevated in Type 2 diabeteics (plasma viscosity: Type 1 diab. mell.:1,28 vs. control:1,27; Type 2 diab. mell.:

1,32±0,10 vs. control: 1,27, p>0,05. Fibrinogen level: Type 1 diab. mell.: 3,14±1,18 vs. control: 2,46±0,90, p>0,05; Type 2 diab. mell.: 3,57±1,86 vs. control: 2,46±0,90 p=0,06). However, *plasma viscosity* in patients with *nephropathy* and *proliferative retinopathy* was significantly higher compared to controls and patients without complications or with background *retinopathy* (nephropathy vs. control p<0,05; proliferative retinopathy vs. control p<0,001).

The level of *vWF* was found to be significantly elevated in Type 2 diabetics.

Homocystein levels were significantly higher in Type 1 diabetics than in the Type 2 group. The association with age has also been proven that is known from literature. There was a correlation with microalbuminuria, but the occurrence of nephropathy was indipendent from hyperhomocysteinaemia.

In patients with *Helicobacter pylori* positivity severe microvascular complications occurred more frequently than in patients without bacterial infection. The ratio of Helicobacter positivity in our group was 65% higher than in the non diabetic group, 55% (the mean age was about the same in both group).

STATEMENTS

The number of *platelet-monocyte heterophilic aggregates* in patients with Type 1 and Type 2 diabetes mellitus and with chronic myeloproliferative disorders with high platelet count is significantly elevated.

There is no association between the balance of carbohydrate metabolism (HgbA1c) and the percentage of heterophilic aggregates.

The highest number of monocyte-platelet heterophilic aggregates was found in patients with *severe microvascular complications* like proliferative retinopathy and nephropathy, but diabetic patients without vascular complications still had significantly higher aggregate numbers compared to the control subjects. The HgbA1c level was significantly higher in the diabetic group (Type 1 diabetes: 9,11±2,60; Type 2 diabetes: 9,32±2,39; control group:5,16±0,21), but no association was found with the number of heterophilic aggregates, and the number of aggregates also seemed to be independent of the duration of disease.

A non significant, but remarkable tendency in the elevation of *postprandial aggregate formation* has been recognized 2 hours after an average breakfast containing 50 g carbohydrates.

Patients with *chronic myeloproliferative disorders* with high platelet count showed nearly twice as high mixed aggregate numbers than diabetic patients. These results show that increased formation of platelet-monocyte aggregate may have a role in the development of diabetic vascular complications and vascular symptomes of patients with different chronic myeloproliferative disorders.

In vitro *platelet NO responses* were reduced in each of the diabetic subjects, the speed of NO generation was also slow with some of the stimulants. One may assume, that blunted platelet NO response, in part, at least, may contribute to platelet hyperfunction and angiopathy in diabetes mellitus.

There was no significant difference found between diabetic and control group in *plasma viscosity,* but *fibrinogen level* was nearly significantly elevated in Type 2 diabeteics. However, *plasma viscosity* in patients with *nephropathy* and *proliferative retinopathy* was significantly higher compared to controls and patients without complications or with background *retinopathy*.
Deformability of red blood cells was derceased significantly in both diabetic groups.

Oxidative stress was increased significantly in both type of diabetes in both, the plasma and in the red blood cells. Increased oxidative stress may alter the structure and the fluidity of the membrane of red blood cells and decrease their deformability.

Among factors contributing to the pathogenesis of diabetic angiopathy the following associations can be set up:

- There was a significant positive correlation between platelet-monocyte and platelet-granulocyte heterophilic aggregate formation. The elevation of platelet-monocyte aggregate number was significant while the elevation of platelet-granulocyte aggregate number was not.
- There was a positive correlation between HgbA1c and oxidative stress in Type 1 diabetics and the duration of diabetes and oxidative stress in Type 2 patients.
- Plasma viscosity showed a positive correlation with the duration of diabetes in Type 1 patients and with HgbA1c and fibrinogen levels in Type 2 diabetics.

According to our results changes in haemorheology and in oxidative stress may have a part in the pathogenesis of diabetic angiopathy.

Homocystein levels were significantly higher in Type 2 patients compared to Type 1 patients and there was an association between hyperhomocysteinaemia and microalbuminuria. It can be calculated that hyperhomocysteinaemia is an important additive factor in the development of angiopathy.

Helicobacter pylori **positivity occured more frequently in the diabetic group. The number of severe microvascular complications was also higher in patients with H.p. positivity. It needs further examinations to decide if Helicobacter positivity has any effect in the pathogensis of diabetic angiopathy.**

PUBLICATIONS

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