Pergamon

Thrombosis Research, Vol. 79, No. 1, pp. 57–63, 1995

0049-3848/95 $9.50 + .00

0049-3848(95)00090-9

ALTERED LYSIS RESISTANCE OF PLATELET-RICH CLOTS IN PATIENTS WITH INSULIN-DEPENDENT DIABETES MELLITUS

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(Received 16 December 1994 by Editor L. Muszbek; revised/accepted 20 April 1995)

Abstract: The fibrinolytic resistance of platelet-rich arterial thrombi received much attention. Clot lysis method was used to assess the *in vitro* fibrinolytic properties in diabetes mellitus. Platelet rich (PRP) clots were formed by addition of thrombin, and lysis was induced by tissue-plasminogen-activator. The coagulation and lysis was followed by the light scattering properties. A special pattern of good initial lysis followed by a second clotting phase was observed in more than half of insulin dependent diabetic patients, while a similar pattern of clot-lysis was only occasionally found in non-insulin dependent diabetes mellitus or in the healthy control group. Following the thrombin activation of washed, gel-filtered platelets, the supernatants possessed an inhibitory action on *in vitro* lysis of PPP-clots. This suppression was remarkably stronger in IDDM, along with the highest PAI-1 activity concentration ratio of the platelet lysates, compared to plasmatic levels. The relation of this special type of PRP clot-lysis resistance to diabetic vascular complications needs further clarifying and investigations.

Micro- and macrovascular complications have an undisputed role and major influence on the lifespan and quality of patients with both insulin dependent (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). An abundant representation of different haemostatic abnormalities, including platelet hyperfunction, altered activity of different coagulation pathways and inhibitors had been described as a possible predecessor, cause or consequence of diabetic vascular disease (1, 2). Diverse fibrinolytic abnormalities were reported using *in vitro* whole blood clot-lysis assays or analysis of separated fibrinolytic components (1, 3, 4, 5) in IDDM and NIDDM as well. The lysis resistance of the principally platelet-rich arterial thrombi, probably as a result of clot-retraction and also due to the presence of fibrinolytic inhibitors in platelets (mainly PAI-1) is in good agreement with general clinical observation and can be detected by

Key words: fibrinolytic resistance, platelet rich clots, diabetes mellitus, plasminogen activator inhibitor

Abbreviations: IDDM; insulin dependent diabetes mellitus, NIDDM; non-insulin dependent diabetes mellitus, OD; optical density, PPP; platelet poor plasma, PRP; platelet rich plasma, V; Δ; OD/min

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as well (6, 7). Platelet abnormalities are frequently described in diabetes mellitus (1, 2, 8, 9), however, few is known about the lysis properties of platelet-rich thrombi in diabetics. In this report an *in vitro* approach was applied to characterize the properties of clot-lysis, composed of platelet-poor (PPP) or platelet-rich (PRP) plasmas in health and in diabetes mellitus.

PATIENTS, MATERIALS AND METHODS

The healthy control group consisted of 24 members of the faculty personnel (doctors, nurses, laboratory workers, students) without known metabolic, vascular or haemostatic disorders. Eleven females and thirteen males were included (21 to 46 years, mean age 33 years, smoker 3).

IDDM group. 16 patients were examined (1o males, 6 females, mean age 28 years, smoker 2).
NIDDM group was composed of 31 patients with diabetes mellitus, they received diet and/or oral antidiabetic treatment (19 females, 12 males, mean age 48 years, smokers 8).

None of the controls or diabetic patients received drugs, which may alter haemostasis or platelet function prior 10 days to the examinations. Both type of diabetics were in good metabolic control at the time of blood sampling. Patients with major vascular or infective complications (diabetic feet, proliferative retinopathy, acute myocardial event or urinary infection, pneumonia, etc.) were not incorporated in the test series.

Venous blood was withdrawn from the antecubital vein employing a clean venipuncture technique between 8 and 10 AM. Anticoagulation was obtained by 3.9% sodium-citrate at a ratio of 9:1. PPP and PRP was gained by differential centrifugation.

Clot-lysis assay mixtures were composed of different combinations of PPP and PRP derived from the control persons or diabetic patients. The estimations were performed as it had been described (10), briefly as follows:
50 µl aliquots of PRPs (platelet count: 45oooo/ml) were clotted in triplicate in a 96 wells microplate (Propilen GMK, Pecs, Hungary) by 5 U/ml thrombin (Topostasin, Roche). Lysis was induced by tissue plasminogen activator (tPA, 2 U/ml final concentration, gift of the Catholic University, Leuven, Belgium, diluted in tris-saline buffer, pH 7.4). The procedure of coagulation and clot-lysis were followed and characterized by the increase and decrease of optical densitiy (OD, registered in five minute intervals), checked by the Microelisa Reader (Twinreader, Labsystems, Helsinki, Finland) at 450 nM wavelength. In our settings the lysis took 60-90 minutes. A lysis speed calculation (change of mOD/min: V) was applied for characterization of the lysis curves. Clot formation and lysis, or the occasional recoagulation was checked visually and mechanically as well.

Influence of the supernatants of activated platelets on clot-lysis. Washed and gel-filtered (Sepharose CL-2B, Pharmacia, Uppsala, Sweden) platelets, resuspended in tris-saline, HEPES, Ca, albumin containing buffer (pH 7.4) were activated by thrombin, aggregation was followed in an aggregometer. After centrifugation aliquots of platelet supernatants (or the washing buffer as blank) were mixed to the PPP clot-lysis assay mixtures (derived from the same individual) to estimate the influence of the lysates of activated platelets on *in vitro* fibrinolysis.

Plasmin generation. 10 μg aliquots of glu-plasminogen (fibrinogen-free, purity was checked by polyacrilamide gel-electrophoresis), separated from plasmapheresis plasma (11) were activated by tPA (4 U/ml), the rate of plasmin generation in the presence or absence of supernatants of the gel-filtered, thrombin activated platelets were assessed by the amidolytic chromogenic reaction (0.7 mM S-2251, Kabi, Stockholm, Sweden) at OD 450, using the microplate ELISA device, according to the recent modification (12).

PAI-1 activity of plasmas and platelet supernatants were evaluated by a commercially available chromogenic method (Boehringer, Mannheim, Germany). The PAI-1 activity of the supernatants were corrected to 100000 platelets/μl platelet count.

RESULTS

The overall clot-lysis results accomplished in PPP and PRP environment, characterized by the *V* values are summarized briefly in Table I. The influence of the supernatants of thrombin activated platelets are also demonstrated. The lysis speed of PRP-clots were significantly lower (paired t tests) in the control group ($p<0.02$) and in both types of diabetes mellitus ($p<0.005$ and 0.05 respectively) comparing with PPP clot-lysis of the identical groups.

The analysis of the individual PRP clot-lysis curves revealed a special pattern of rapid initial lysis in the first 30 min, followed by a recoagulation phase in 10 out of 16 IDDM patients. This biphasic pattern was also observed in 4 out of the 31 NIDDM patients and in one individual of the control group. The bimodal lysis curve was significantly more frequent in IDDM, than in all the other groups (non-parametric chi-square test, $p<0.01$). A typical appearance of quick PRP-lysis continued by a recoagulation is illustrated in Fig.1. Biphasic lysis of PRP clots in IDDM was also observed if streptokinase was used instead of tPA.

The PAI-1 activity (U/ml) of PPP and in the supernatants of activated platelets are depicted in Table II.

TABLE. I

In vitro Clot-lysis and its Modification by the Supernatants of Activated Platelets

	V±SD, PPP	V±SD, PRP	V±SD, PPP±supernatant
Control (n=24)	7.11±2.03	4.16±1.89	4.89±2.21
IDDM (n=16)	11.27±5.64	5.22±2.35	4.35+2.97
NIDDM (n=31)	4.39±1.93	2.87±0.98	3.11± 1.17

TABLE. II
PAI-1 Activity Measured in PPP and in Supernatants of Thrombin Activated Gel-filtered Platelets

	PAI-1±SD PPP	PAI-1±SD supernatant	PPP/ supernatant PAI-1 ratio
Control (n=24)	8.14±2.25	3.45±1.19	2.36
IDDM (n=16)	7.21±3.12	6.88±2.85	1.18
NIDDM (n=31)	16.56±6.28	7.76±3.44	2.28

PPP PAI-1 activity was similar in the control and IDDM group, but the activity was significantly elevated in NIDDM ($p<0.002$). The absolute concentration of PAI-1 after the release of thrombin activated gel-filtered platelets proved to be somewhat elevated ($p<0,05$) in diabetics, whereas the ratio of PPP PAI-1 activities to platelet supernatant level was identical in the control and NIDDM patients. The PAI-1 activity of the platelet supernatants was also elevated in IDDM ($p<0.05$) comparing with the control group, moreover the ratio to PPP PAI-1 activity seemed to be remarkably low.

The results gained by chromogenic assays also revealed inhibition of plasmin generation in the presence of the supernatants yielded from gel-filtered, thrombin activated platelets. This more than two-fold suppression of the initial velocity of plasmin generation was strikingly prominent in case of IDDM platelet supernatants. Fig. 2. illustrates the typical pattern of the chromogenic reaction in a representative series of examination done in a control (supernatant of platelets of a control person), IDDM and NIDDM patient. Similar results were gained with 6 other series of measurements, the mean reduction of plasmin generation velocity was 2.12+o,86 (expressed in *V* value) if IDDM supernatants were used.

DISCUSSION

Different approaches provided by several *in vitro* whole blood- and fibrin-clot lysis methods might be suitable for modelling some steps of fibrinolysis (10). The lysis-resistance of platelet-rich clots could have been well registered in the healthy control group with the method applied here, the results are in good agreement with the clinical experience and with previously published *in vitro* experimental data (6).

A wide spectrum of fibrinolytic abnormalities - including some conflicting data - had been described in diabetes mellitus, i.e. altered fibrinogen structure (13), facilitated lysis, probably induced by insulin itself in IDDM (14) or reduced fibrinolysis in NIDDM (15). The present investigation of PPP-clots seems to provide some further evidence, that reduced fibrinolysis occurs more frequently in NIDDM. However, less is known about the lysis resistance of platelet-rich clots in diabetes mellitus (16). Platelet alpha granules contain a substantial amount of PAI-1. Nevertheless, PAI-1 released from freezed-thawed platelets possesses only moderate inhibitory activity (17), but if platelets of NIDDM patients were examined, the PAI-1

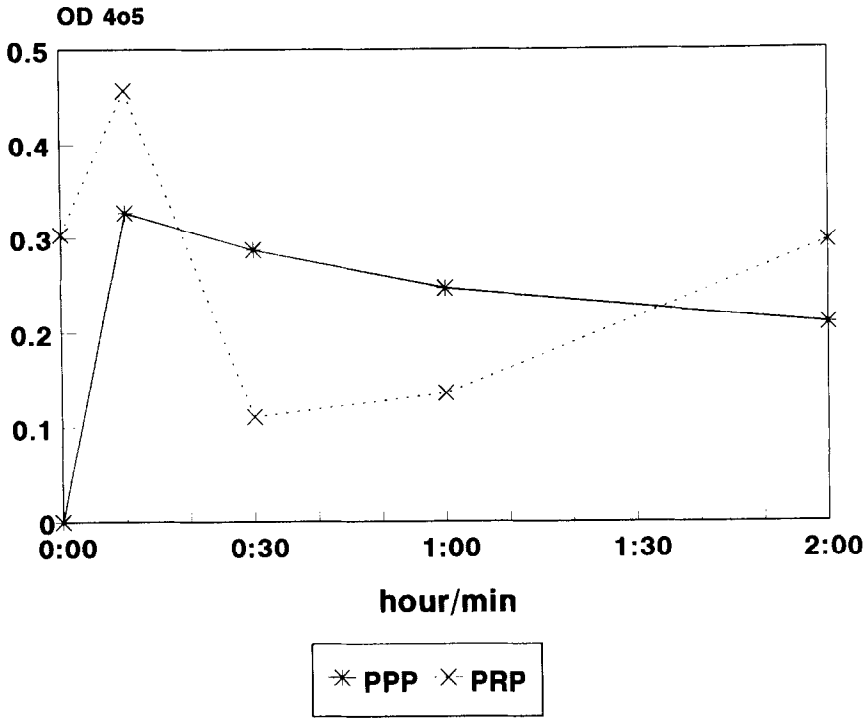

FIG. 1

Biphasic clot-lysis in IDDM

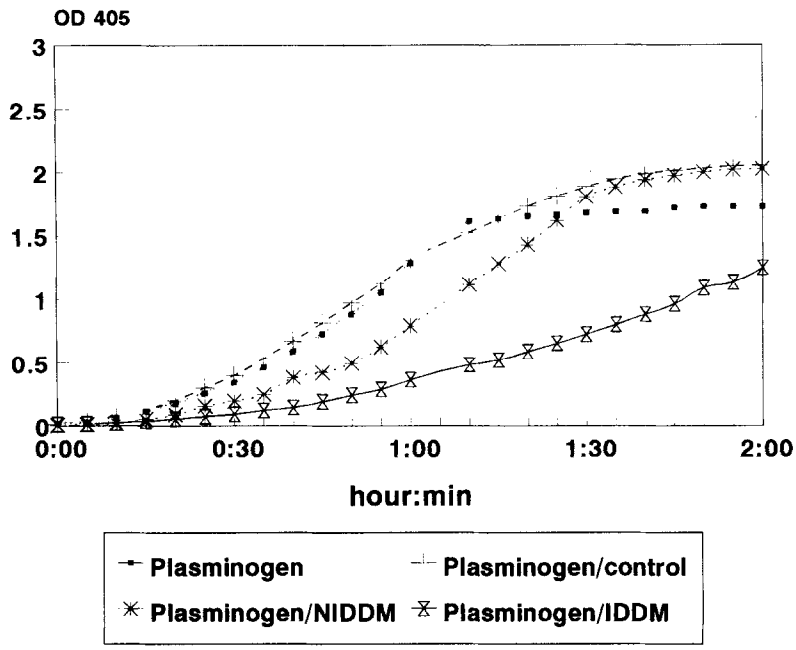

FIG. 2

Plasmin generation in the presence of supernatants of activated platelets

activity seemed to relatively preserved or even raised (18).

In our settings platelet-rich composition of clots resulted in stronger lysis-resistance in both types of diabetes mellitus comparing with the control group. The resistance was even more accentuated in IDDM cases, and a special pattern of rapid initial lysis, followed by a second coagulatory phase was detected in more than half of IDDM patients. Anyway, this may not be regarded as a unique finding, some cases in NIDDM and even one among healthy persons were also registered. This bimodal fashioned lysis of platelet-enriched clots has also been described previously (6). We were not able to find any correlation to the length of diabetes, metabolic control, vasculopathy, etc. However the findings were consequently registered if the lysis experiments were repeated several days or weeks later (using blood samples withdrawn at different occasions). Further efforts seem to be necessary to elucidate the correct relation and impact of this phenomenon to platelet abnormalities and vascular complications observed in IDDM.

The PAI-1 activity harvested from washed, gel-filtered, thrombin activated platelet supernatants was elevated in both type of diabetes mellitus. In the presence of this supernatants clot lysis was markedly inhibited in IDDM patients. As the same supernatants were also capable to retard chromogenic plasmin generation induced by tPA, it seems reasonable to suggest, that the inhibition caused by the supernatants of activated platelets can be attributed mainly to their PAI-1 content. This inhibition was specially remarkable and accentuated in IDDM.

Platelet PAI-1 is considered to be mainly inactive, if freeze-thawed or triton solubilized platelet lysates were assayed (17). Probably the activation of platelets by thrombin is able to modify PAI-1 activity. The data presented here suggest, that the platelets of IDDM patients can cause a special type of in vitro clot lysis resistance, probably due to more active platelets (and probably higher activity of platelet PAI-1) in IDDM.

Acknowledgements

The laboratory assistance of Ms Maria Fiak is highly appreciated, along with the help of Ms Erzsebet Major in preparing the manuscript.

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