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Analysis of platelet deposition and binding of anti-fibrinogen antibody to the artificial surfaces preadsorbed with sera or plasmas from patients with normal and elevated CRP

Analiza adhezji płytek krwi i wiązania przeciwciał rozpoznających fibrynogen do sztucznych powierzchni opłaszczonych surowicą lub osoczem pacjentów z prawidłowym i podwyższonym stężeniem białka C-reaktywnego

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Summary

Background: As far as fibrinogen (Fb) is an essential protein in mediating platelet adhesion *in vitro*, the colorimetric adhesion assay can differentiate platelet deposition to normal plasma from fibrinogen-deficient plasma in healthy donors. However, it is not known whether it may discriminate platelet response to serum and/or plasma originating from patients with and without the increased inflammatory activity.

Aims: In the present study we compared ADP-induced platelet adhesion in healthy subjects to serum or plasma from patients with normal and increased CRP concentration (CRP>5 mg/L). Also, the binding of anti-Fb antibodies to serum and plasma from patients with varying CRP concentrations was examined.

Methods: For microplate coating, both serum and plasma from patients with varying CRP concentrations were utilized. Colorimetric assays were used to determine platelet adhesion and anti-Fb antibody binding.

Results: We demonstrated significantly higher platelet adhesion to the sera and plasmas from patients with elevated CRP compared to platelet adhesion to the sera and plasmas from patients with normal CRP. Also, we observed significantly higher anti-Fb binding to the sera from patients with elevated CRP compared to anti-Fb binding to the sera from patients with normal CRP. Furthermore, the binding of anti-Fb antibody to the sera of patients significantly correlated with CRP concentration.

Conclusions: Overall, this study supports the data on the existence of a strong relation between CRP and fibrinogen level and it leads to the conclusion that the adhesion assay may be a useful tool in differentiating platelet response to sera and plasmas from patients with and without the increased inflammatory activity.

Streszczenie

Wstęp: Fibrynogen (Fb) jest kluczowym białkiem biorącym udział w adhezji płytek krwi w warunkach *in vitro*, dlatego też kolorymetryczny test pomiaru adhezji płytek krwi pozwala różnicować adhezję płytek krwi do osocza z prawidłowym poziomem fibrynogenu od adhezji płytek krwi do osocza deficytowego pod względem fibrynogenu. Nie wiadomo jednak, czy test adhezji może dyskryminować adhezję płytek do surowicy i/lub osocza pochodzących od pacjentów ze/bez zwiększoną aktywnością zapalną, definiowaną stężeniem białka C-reaktywnego (CRP).

Cele: W badaniu porównywano adhezję płytek krwi stymulowanych ADP (izolowanych od zdrowych dawców) do surowicy lub osocza pacjentów z prawidłowym lub podwyższonym (CRP>5 mg/l) stężeniem CRP. Ponadto, oznaczano wiązanie przeciwciał anty-Fb do surowicy lub osocza pochodzących od pacjentów z prawidłowym/podwyższonym stężeniem CRP.

Metody: Do opłaszczania mikropłytek wykorzystano surowicę i osocze pochodzące od pacjentów z prawidłowym lub podwyższonym stężeniem CRP. Testy kolorymetryczne posłużyły do oznaczenia adhezji płytek krwi i oceny wiązania przeciwciał anty-Fb.

Wyniki: Wykazano znacząco wyższą adhezję płytek krwi do surowic i osocz pacjentów z podwyższonym stężeniem CRP, w porównaniu do adhezji płytek krwi do surowic i osocz pacjentów z prawidłowym poziomem CRP. Zaobserwowano również istotnie wyższe wiązanie przeciwciał anty-Fb do surowic pacjentów z podwyższonym stężeniem CRP, w porównaniu do wiązania anty-Fb do surowic pacjentów z prawidłowym poziomem CRP. Ponadto, wiązanie przeciwciał anty-Fb do surowicy pacjentów korelowało ze stężeniem CRP w surowicy.

Wnioski: Powyższe badania wspierają dane opisujące zależność między stężeniem CRP i fibrynogenu w osoczu i sugerują, że zastosowany test adhezji może być użytecznym narzędziem w różnicowaniu adhezji płytek krwi do surowic lub osoczy pochodzących od pacjentów ze/bez zwiększoną aktywnością zapalną.

Key words: antibody binding, C-reactive protein, fibrinogen, platelet adhesion, protein adsorption
Słowa kluczowe: adhezja płytek, adsorpcja białek, białko C-reaktywne, fibrynogen, wiązanie przeciwciał

Abbreviations: ADP; adenosine 5′-diphosphate sodium salt, AP; alkaline phosphatase, anti-Fb; antibody anti-fibrinogen, BSA; bovine serum albumin, CRP; C-reactive protein, ELISA; enzyme-linked immunosorbent assay, Fb; fibrinogen, HRP; horseradish peroxidase, HSA; human serum albumin, pNPP; p-nitrophenylphosphate, PBS; phosphate buffered saline, PRP; platelet-rich plasma, TMB; 3,3′,5,5′-tetramethylbenzidine, TBS; tris-buffered saline, TBST; tris-buffered saline with Tween 20; BCA method, bicinchoninic acid method.

Introduction

Platelet adhesion to the surfaces coated with fibrinogen or collagen provides a simple and useful model for studying platelet function and the receptorial mechanisms involved in the regulation of platelet response *in vivo* [1-3]. Sometimes, in experiments of platelet deposition, other biologically important proteins, derived from plasma or extracellular matrix, are used for coating purpose. For example, C1q, the initiating protein of the classical complement cascade, has been employed to examine the function of platelet membrane C1q receptors [4]. Eriksson et al. [5] immobilized albumin onto artificial surfaces in order to resolve the mechanisms of the synergistic effects of signal molecules, such as adrenaline and lysophosphatidic acid (LPA) on static platelet adhesion. Likewise, Brennan et al. [6] used purified C-reactive protein (CRP) for coating artificial surfaces to investigate the contribution of $\alpha_{IIb}\beta_3$ in platelet deposition to CRP. When the adhesiveness of blood platelets on artificial surfaces is considered, the non-coated surfaces are utilized [7, 8]. According to our recent findings, deposition of platelets in platelet-rich plasma to non-coated polystyrene plates can be extremely high [9].

A meaningful platelet deposition, similar to that observed with purified fibrinogen, can be obtained on the surfaces immobilized with supernatant from platelet concentrates activated with platelet agonist (the so-called activated plasma) [5, 10] or preadsorbed with plasma [11], in which fibrinogen plays essential role in mediating platelet adhesion. The contribution of fibrinogen in platelet adhesion to polymers or glass surfaces was mostly evidenced by comparison of the effect of plasma dilution on fibrinogen adsorption and platelet adhesion, and also by the use of deficient plasma. Zucker et al. has observed that the adsorption of fibrinogen is a prerequisite for platelet adhesion to glass as the cells adhered to glass slides when they were previously exposed to normal plasma or fibrinogen but not to slides exposed to serum and afibrinogenemic plasma [12]. Similar observations were obtained in the studies of baboon fibrinogen adsorption and platelet adhesion to polyetherurethane materials [13]. Platelet adhesion was almost completely inhibited when baboon or human plasmas lacking fibrinogen were used (i.e. serum, defibrinogenated plasma, and congenitally afibrinogenemic plasma), but it was restored to near normal after adding exogenous fibrinogen to fibrinogen-deficient plasmas [13]. In the further studies, the γ-chain C-terminal dodecapeptide in fibrinogen molecule has been shown to be the most important site in adsorbed fibrinogen, responsible for platelet adhesion [14]. Fibrinogen itself was also revealed to adsorb more strongly than most other blood proteins to many surfaces, especially to the polymeric ones [15]. Based on above findings, it can be concluded that the method of platelet adhesion discriminates platelet deposition to normal plasma from that to fibrinogen-deficient plasma or serum in healthy subjects. However, it is not known whether the method of platelet adhesion may differentiate the response of platelets from healthy subjects to serum or plasma originating from patients with normal or increased inflammatory activity, defined by the level of C-reactive protein (CRP 5 mg/L and more).

Both fibrinogen and CRP are acute phase proteins and their concentrations increase during inflammation caused by infection, tissue injury or chronic disease. Moreover, they are a significant clinical tool in cardiovascular risk stratification and cardiovascular disease prediction [16, 17]. The levels of these CVD predictors are, to a large extent, genetically determined and there is a significant correlation between CRP and fibrinogen levels [18, 19]. Therefore, the aim of this study was to compare platelet adhesion in healthy subjects to polymer surfaces preadsorbed with serum and plasma derived from patients with normal and elevated CRP concentration. Moreover, we evaluated the binding of anti-fibrinogen antibodies to serum and plasma of patients with normal and elevated CRP. Our results suggest that platelet adhesion assay may be a useful tool in differentiating platelet adhesion to sera/plasmas from patients with and without the increased inflammatory activity.

Materials and Methods

Chemicals

C-reactive protein (CRP; purity >97%) was obtained from the Binding Site (Birmingham, UK). Goat polyclonal antibodies directed against human fibrinogen conjugated with horseradish peroxidase (HRP) and the Micro BCA™ Protein Assay Kit were from Thermo Scientific (Rockford, IL, USA). Phosphate buffered saline (PBS), pH 7.4, was from Biomed (Lublin, Poland). Sterile saline solution (0.9%) was from Polpharma (Warsaw, Poland). Magnesium chloride and calcium chloride were from POCH (Gliwice, Poland). Human serum albumin (HSA; purity 96–99%), bovine serum albumin (BSA; purity ≥98%), fibrinogen (purity 50-70%), p-nitrophenylphosphate (pNPP), 3,3′,5,5′-tetramethylbenzidine (TMB), adenosine 5′-diphosphate sodium salt (ADP), polyoxyethylene(20)sorbitan monolaurate (Tween 20), Triton X-100 and other chemicals were from Sigma (St. Louis, MO, USA), unless otherwise stated. Polystyrene 96-well flat-bottom microplates were from Nunc (MaxiSorp) (Waltham, MA, USA).

Table I. Characteristics of biological material immobilized on polystyrene plates in experiments of platelet adhesion and anti-Fb binding.

Parameter	Serum			Plasma		
	Control subjects CRP < 5 mg/L n=15	Patients CRP < 5 mg/L n=16	Patients CRP > 5 mg/L n=16	Control subjects CRP < 5 mg/L n=15	Patients CRP < 5 mg/L n=16	Patients CRP > 5 mg/L n=16
gender [F/M]	12/3	9/7	5/11	12/3	6/10	4/12
age [yrs]	33 ± 12	66 ± 18	69 ± 14	33 ± 12	56 ± 16	67 ± 19
CRP (mg/L)	0.4 [0.2–1.6]	1.3 [0.8–1.9]	118.2 [30–260]	0.4 [0.2–1.6]	2.0 [0.7–3.6]	28.3 [17.1–56.1]

Selection of participants and framework of the study

For platelet adhesion study, the blood was drawn from 16 healthy donors (3 men and 13 women; mean age 27 ± 6 years) into polypropylene 0.105 M sodium citrate tubes. None of the donors had taken aspirin or other drugs affecting platelet function for at least 14 days prior to blood collection. The study was approved by the Human Studies Committee of the Medical University of Lodz (Poland) and was conducted in accordance with guidelines established by the Declaration of Helsinki.

Most observations are based on the experiments of platelet adhesion to serum and plasma immobilized on polystyrene wells and originating from healthy individuals (control experiments) and hospitalized patients with varying CRP concentrations. Platelet adhesion to purified plasma proteins (fibrinogen, CRP, HSA) was also measured. A more detailed description of biological material used in the study is given in Table I.

Diagnostic determination of CRP in human samples

High-sensitivity CRP concentration (normal range below 5 mg/L) in serum samples was determined by immunoturbidimetric method on an Olympus analyzer (Beckman Coulter, Warsaw, Poland).

Preparation of isolated platelets

Blood was withdrawn into the one-seventh volume of buffered 0.105 M sodium citrate in a polypropylene tube and immediately centrifuged for 12 min at 190 x g to obtain platelet-rich plasma (PRP). For platelet isolation, PRP was centrifuged at 800 x g for 12 min to sediment platelets. Then, the supernatant was removed and platelets in the pellet were resuspended in Tyrode buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM glucose, 0.2% BSA, pH 7.4) and adjusted to 1 x 10^8 per mL.

Platelet adhesion assay

Platelet adhesion assay was performed according to the method of Eriksson et al. [10]. Briefly, 100 µL aliquots of 1% human serum or 1% plasma with known CRP concentrations were added to the wells in 96-well microplates and the plates were incubated overnight at 4°C without mixing. The concentration of biological material was based on the previous observation indicating that intermediate dilutions of plasma (0.1-1%) are optimal for efficient fibrinogen adsorption [20] and platelet adhesion [2]. Alternatively, the wells of polystyrene plates were coated with increasing concentrations of purified fibrinogen and HSA as a control up to 1.5 mg/mL and immobilized overnight at 4°C without mixing. On the following day, wells were washed twice with 0.9% saline and blocked with 0.2% BSA (1h, 37°C). After washing, 50 µL of platelet suspension (1 x 10^8 platelets/mL) and 50 µl of Tyrode buffer containing 20 µM ADP, 10 mM $CaCl_2$ and 10 mM $MgCl_2$ were added to each well and the microplates were incubated for 1h without shaking. The wells were then washed twice with saline to remove unattached platelets and the wells were rapidly filled up with 150 µL of substrate solution (0.1 M citrate buffer, pH 5.4, containing 1 mg/mL pNPP and 0.1% Triton X-100). For the estimation of what we referred to as "a total platelet adhesion", 50 µL of platelet suspension was mixed with 150 µL of substrate solution and added to an uncoated microplate; 50 µL of Tyrode buffer was used as blank. The microplates were incubated with substrate solution for 1h at 37°C under gentle shaking and reaction was stopped by the addition of 100 µL of 2N NaOH. The colored *p*-nitrophenol, produced in the reaction, was measured by absorbance reading at 405 nm. The percentage of adherent cells was calculated using the formula: (sample–blank)/(total–blank) × 100.

Binding of polyclonal anti-fibrinogen antibodies to serum or plasma with known CRP concentrations

The wells of 96-well microtiter plates were coated overnight at 4°C with 1% single donor and pooled serum/plasma with the known CRP concentrations (100 µL per well). Unbound protein was removed by washing with Tris-buffered saline (TBS), pH 7.4, and the wells were blocked with 1% BSA in TBS containing 0.05% Tween 20 (TBST) for 1h at 37°C. After blocking, the wells were washed three times with TBST and incubated with antibodies against human fibrinogen (anti-Fb/HRP 1:100,000) in TBST for 1h at 37°C. All wells were washed again and 100 µL of substrate solution (0.1 mg/mL TMB in citrate buffer with hydrogen peroxide, pH 5.4) was added to each well and incubated for 30 min at 37°C in the dark. Then, the reaction was stopped by adding 50 µL of 0.5 M H_2SO_4 and the optical density was measured at a wavelength of 450 nm.

Protein assay

Protein content (the amount of protein adsorbed) in the wells coated with purified fibrinogen at the concentration range of 0.02-1.5 mg/mL was assessed as reported previously [21]. The BCA assay was performed according to the manufacturer's instructions for protein analysis on microplate wells without mixing during immobilization and incubation periods. Briefly, after protein immobilization (2h, 37°C), the wells were washed three times with PBS (pH 7.4) and the amount of protein adsorbed on the polysty-

rene plates was measured using a Micro BCA™ Protein Assay Kit, which gives a linear relationship in the range of 0.5 to 20 µg/mL protein. The amount of protein adsorbed on the microwells was determined using a standard curve for bovine serum albumin (BSA) over the range of 0.5-2 µg/well.

Data analysis

CRP concentrations are expressed as median and interquartile range. The remaining results are expressed as arithmetic mean ± standard error (SE). Normality and homogeneity of variances were verified with the Shapiro-Wilk's test and the Levene's test, respectively. The raw data were analyzed using the unpaired t test. Platelet adhesion to the sera and plasmas derived from patients with normal or increased CRP concentrations was additionally analyzed by ANCOVA using the CRP concentration and anti-fibrinogen binding as covariates. For the assessment of maximum platelet adhesion to the purified proteins, the dose-response curves were plotted using the nonlinear regression analysis and the B_{max} values were calculated with GraphPad Prism software (v. 5.03). The Pearson's linear correlation was used to assess associations among the measured variables.

Results

Platelet adhesion to purified fibrinogen

Before determining platelet adhesion to the sera and plasmas of patients with normal and increased inflammatory activity, we assessed adsorption of purified fibrinogen on polystyrene plate and platelet deposition on the wells coated with purified fibrinogen in the concentration range of 0.02-1.5 mg/mL.

ADP-induced platelet adhesion to fibrinogen was significantly higher from the platelet adhesion on HSA-coated wells (respectively 41.0 ± 5.8% vs. 2.5 ± 0.2%, p=0.004; n=5). In contrast to HSA, the platelet adhesion to fibrinogen increased gradually with increasing concentrations of immobilized protein (R_P=0.54, p=0.0007) and the amount of fibrinogen adsorbed (R_P=0.77, p<0.05).

Platelet adhesion to the surfaces coated with the sera and plasmas from patients with varying CRP concentrations and healthy donors

CRP concentration in the serum samples derived from patients with normal and augmented CRP was 1.3 [0.8–1.9] mg/L (n=16) and 118.2 [30–260] mg/L (n=16), whereas in the plasma samples from patients with normal and elevated CRP it was 2.0 [0.7–3.6] mg/L (n=16) and 28.3 [17.1–56.1] mg/L (n=16). The concentration of CRP in the samples of serum and plasma from healthy volunteers was 0.4 [0.2–1.6] mg/L (n=15). Platelet adhesion to the sera from patients with elevated CRP concentration was nearly 2-fold higher from that measured on the plate preadsorbed with the sera from patients with normal CRP (p<0.01 by the unpaired t-test, p<0.05 by ANCOVA with CRP and anti-Fb binding as covariates; n=3 experiments on 16 sera from patients with normal CRP and 16 sera from patients with elevated CRP) (Fig. 1A). Similarly to this, when the microtiter plates were preadsorbed with plasmas, we observed 1.3-fold higher platelet adhesion to the plasmas from patients with elevated CRP concentration than to the plasmas from patients with normal CRP (p<0.001 by the unpaired t-test, p<0.001 by ANCOVA with CRP and anti-Fb binding as covariates; n=3 experiments on 16 plasmas from patients with normal CRP and 16 plasmas from patients with elevated CRP) (Fig. 1A).

Also, there was a significant difference between platelet adhesion to the wells coated with the plasmas from healthy individuals and platelet adhesion to the wells coated with the sera from healthy individuals (res. 19.7 ± 0.5%, vs. 7.6 ± 0.6%, p<0.0001; n=5 experiments on 15 sera from healthy donors and 15 plasmas from healthy donors).

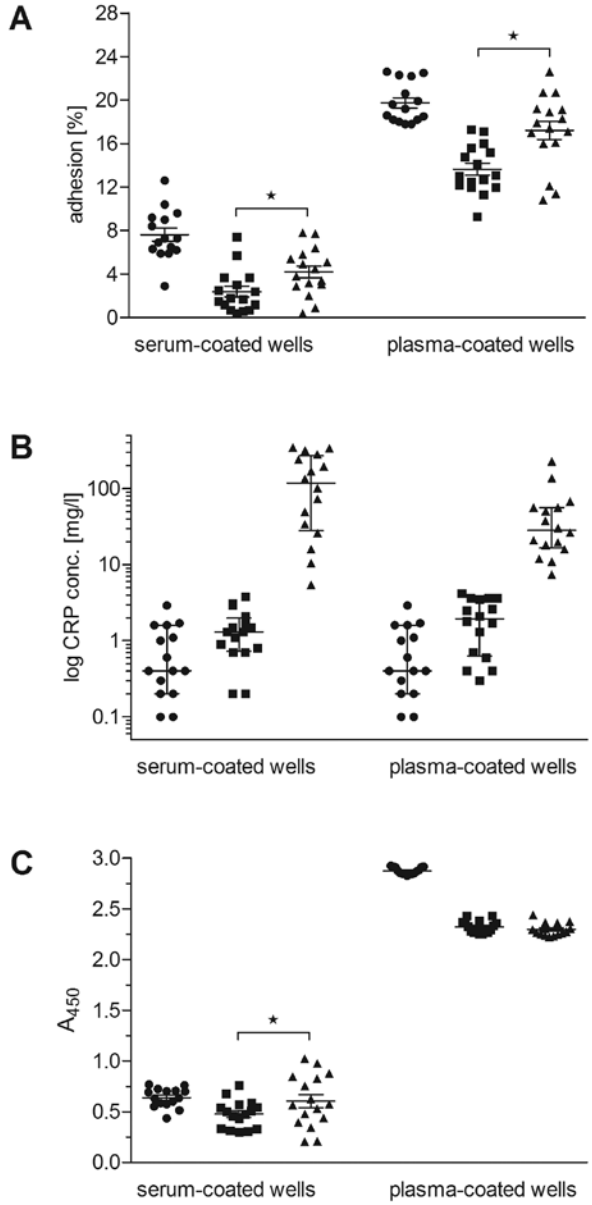

Figure 1. Platelet adhesion, CRP distribution, and binding of anti-fibrinogen antibody to the wells coated with the individual sera and plasmas. All data were collected for three independent groups: healthy subjects (•), patients with normal CRP (■), and patients with elevated CRP (p). Statistically significant differences (P<0.05) between adhesion to the individual sera or plasmas from patients with normal and elevated CRP concentration (unpaired t-test) are indicated by asterisks.

A) Adhesion of isolated platelets stimulated by 10 mM ADP was measured on polystyrene plates preadsorbed with 1% sera/plasmas from control subjects, patients with normal CRP, and patients with elevated CRP level. Data are given as mean ± SE (n=3). B) Distribution of CRP concentration in healthy individuals and patients from whom sera and plasmas were collected. C) The binding of polyclonal anti-Fb antibody to 1% sera and plasmas from healthy subjects, patients with normal CRP, and patients with elevated CRP. The experiment was performed in triplicates for at least 15 individuals within each group.

As shown in Figure 1A, platelet adhesion to the sera and plasmas increased in the following order of serum/plasma donor groups: patients with normal CRP< patients with elevated CRP< control subjects.

Furthermore, we found no significant association between platelet adhesion to the single samples of serum (or plasma) and CRP concentration among the groups of patients and control subjects (Fig. 1A-B).

The binding of anti-fibrinogen antibody to the sera and plasmas from patients with varying CRP concentrations and healthy donors

Fibrinogen concentration in the individual sera/plasma samples used in the experiments was not determined. Instead of it, using the ELISA technique, we investigated the binding of anti-fibrinogen antibody to the surfaces preadsorbed with the sera/plasmas from control subjects and patients with normal and elevated CRP concentration. By the use of polyclonal antibody to human fibrinogen we were able to define the relative adsorption of fibrinogen from the plasmas and sera samples. In preliminary ELISA experiments, using increasing concentrations of purified fibrinogen for immobilization, we confirmed dose-dependent, specific interaction of anti-fibrinogen antibody with its antigen (data not shown).

The binding of anti-fibrinogen antibody to the sera from patients with elevated CRP concentration was significantly higher than that examined on the plate preadsorbed with the sera from patients with normal CRP (absorbance increased 1.5-fold, $p<0.05$, $n=16$) (Fig. 1C). Conversely, the anti-fibrinogen antibody showed similar binding to the plasmas from patients with normal and increased CRP concentration. In control subjects, in which we compared the anti-fibrinogen binding to the individual sera and plasmas, we demonstrated 4.5-fold higher binding of anti-fibrinogen antibody to plasma than to serum ($p<0.0001$, $n=15$) (Fig. 1C).

In the sera of patients with normal and increased CRP concentration, the anti-fibrinogen binding was significantly correlated with CRP concentration (resp. $R_p=0.56$, $p<0.025$ and $R_p=0.62$, $p=0.01$). In the plasmas of patients with normal and increased CRP we also observed a positive correlation between anti-fibrinogen binding and CRP concentration, although it was statistically significant only in patients with normal CRP (resp. $R_p=0.58$, $p<0.02$). Conversely, there was no association between the binding of anti-fibrinogen antibody to the sera and plasmas from healthy individuals and CRP concentration in healthy individuals. Furthermore, platelet adhesion to the surfaces coated with the sera and plasmas from each group (control subjects, patients with normal CRP, patients with elevated CRP) increased with enhanced anti-fibrinogen binding, although the statistically significant correlation was found only in the wells coated with sera of patients with normal CRP concentration ($R_p=0.63$, $p<0.01$).

Discussion

Platelet adhesion assay is a widely used laboratory test for the *in vitro* measurement of functional state of blood platelets and it has been successfully applied for evaluation of changes in platelet response in some inflammatory diseases. For example, the enhanced platelet adherence to virally-infected endothelial cell monolayers has been reported by Visser et al [22]. With the use of cone and platelet analyzer (CPA assay), Knobler et al. showed that patients with noninsulin-dependent diabetes mellitus (NIDDM) may exhibit enhanced deposition and aggregation of platelets on extracellular matrix under flow conditions compared to nondiabetic subjects [23]. The CPA test has been also found to be a valuable method in differentiating patients with antiphospholipid syndrome (APS) with and without thrombotic complications as the platelet response in APS patients with thrombotic events has been demonstrated to be significantly higher compared with APS patients with no thrombosis [24]. Furthermore, the colorimetric adhesion assay on the microtiter plates coated with an autologous serum was employed to detect the changes in equine platelet function which may occur in equine diseases associated with systemic activation of inflammatory cells [25]. This simple assay can also discriminate platelet deposition to normal plasma from fibrinogen-deficient plasma or serum in healthy donors as fibrinogen is essential protein in mediating platelet adhesion *in vitro* [11-13]. However, it is not known whether the platelet adhesion method may differentiate platelet adhesion in healthy donors to serum and/or plasma originating from patients with and without the increased inflammatory activity.

Thus, in the present study we compared the ADP-induced platelet adhesion in healthy subjects to serum or plasma from patients with normal and increased CRP (CRP>5 mg/L). The sera and plasmas from healthy individuals served as a control material in evaluation of platelet deposition. Our results revealed the significantly higher platelet adhesion to the sera and plasmas from patients with elevated CRP compared to platelet adhesion to the sera and plasmas from patients with normal CRP. This leads to the conclusion that the colorimetric adhesion assay may be a useful tool in differentiating platelet response to the serum/plasma of patients with the increased inflammatory activity from that in patients without the increased inflammatory activity.

Along with experiments of platelet adhesion to the sera and plasmas from patients with normal and increased inflammatory activity, we examined the binding of anti-fibrinogen antibody to the wells preadsorbed with serum/plasma from patients with varying CRP. By using polyclonal antibodies, we could analyze the adsorption of fibrinogen from plasma samples and from serum, in which only trace amounts of fibrinogen are present. In general, the data on antibody binding were not surprising, as the binding of anti-Fb antibody to serum was several-fold lower than to plasma. However, in serum-, but not in plasma-coated wells, we found the significantly higher anti-fibrinogen binding to the sera from patients with increased CRP than to the sera from patients with normal CRP. It means, that when the amount of fibrinogen loaded and adsorbed is relatively low, the changes in anti-fibrinogen binding can better reflect the changes in platelet deposition, which were observed on the surfaces coated with sera from patients with normal and increased inflammatory activity. Otherwise, i.e., at the relatively high amount of fibrinogen adsorbed from plasma, we were not able to detect the differences in the anti-fibrinogen binding to the plasma samples from pa-

tients with normal and increased CRP. Similar binding of anti-Fb to plasma of patients with normal and increased inflammatory activity could be a result of comparable adsorption of plasma fibrinogen on polystyrene plates. It is possible because each well on the polystyrene plate has a defined binding capacity (for Nunc microwells it is 100-400 ng/cm^2) [21]. In consequence, at a lower concentration of protein, the amount of adsorbed protein corresponds to the amount of protein added (what we could observe after immobilization of purified fibrinogen), but at a higher protein concentration, the rate of adsorption reaches a plateau. In addition, we immobilized a mixture of plasma proteins. As a result, loading plasma samples with varying fibrinogen concentrations into the well, could lead to similar fibrinogen adsorption and comparable antibody binding. Another reason accounting for the lack of differences in the binding of anti-Fb to the plasmas from patients with normal and increased CRP could be an insufficient antibody concentration with respect to the amount of fibrinogen adsorbed from 1% plasma. On the other hand, in the wells coated with the plasmas from healthy donors, we showed 1.2-fold higher anti-fibrinogen binding than to the wells immobilized with the individual plasmas from patients with varying CRP. It can suggest that there was some excess of antibody over the binding sites.

It should be underlined here that sufficient fibrinogen adsorption is not the only prerequisite for efficient platelet deposition on artificial surfaces. It has been shown that platelet adhesion to polymeric materials may not well correlate with the amount of fibrinogen being adsorbed, and the degree of subsequent platelet adhesion is rather dictated by the conformation of adsorbed fibrinogen [11, 13, 26, 27]. Considering this, we made an attempt to examine the relations between platelet adhesion and the binding of anti-fibrinogen antibody (as an indicator of adsorbed "functional" fibrinogen) to the sera and plasmas from patients with/without the increased inflammatory activity and from healthy donors. Our data seem to support the results of above reports. Whereas we observed a positive correlation between platelet adhesion and anti-fibrinogen binding to the sera and plasmas from patients or from healthy subjects, the statistical significance was only reached in sera of patients with normal CRP concentration. We also demonstrated an association between the CRP concentration and the anti-fibrinogen binding to the sera and plasmas from patients with varying CRP concentrations but we did not reveal such relationship with regard to the sera and plasmas from healthy subjects. This could be due to the lower and aligned concentrations of CRP (and most likely fibrinogen) in healthy subjects, compared to the concentrations of CRP in patients.

To provide possibly high fibrinogen adsorption from biological samples, we used 1/100 serum/plasma dilution (1% serum/plasma), because a peak in fibrinogen adsorption occurs at the intermediate plasma dilution (0.1 – 1%) [2]. The conformation of fibrinogen, like other proteins, usually undergoes some changes upon immobilization on artificial surfaces but to ensure the efficient platelet deposition we used the plates with high binding capacity. According to our earlier data collected on various polymer surfaces [9], the MaxiSorp microplates, which were used in this study, were adequate artificial surfaces for this kind of experiments.

In summary, the core of our study was the analysis of platelet deposition on the artificial surfaces preadsorbed with the sera or plasmas from patients with normal and elevated CRP. Our data indicate that the adhesion assay may be a useful tool in differentiating platelet response to sera/plasmas from patients with and without the increased inflammatory activity. The experiments on platelet deposition and binding of anti-fibrinogen antibody on serum– and plasma-coated wells support earlier observations on the importance of fibrinogen in mediating platelet adherence to artificial surfaces exposed to blood plasma and on the existence of a strong correlation between CRP and fibrinogen level.

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