Modification of human platelet adhesion on biomaterial surfaces by protein preadsorption under static and flow conditions

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Biomaterial-induced thrombosis remains one of the main complications of vascular implant devices. Preadsorbed proteins on the biomaterial/blood interface will modify the adhesion and activation of platelets (PTLs) during the initial contact-phase. Our results clearly show that PTL-adherence on biomaterials is influenced not only by protein preadsorption, but also by flow conditions. The covalent coating of TCPS and glass by phosphorylcholine (PC) induces a significant decrease of PTL adhesion but leads to a slight, but nevertheless significant activation of PTL, which was detected by the induction of P-selectin expression using FACS analysis. Methodologically, the visualization of PTL adhesion gave more reliable results for measurement of PTL adhesion than the cell-enzyme immunoassay (EIA) for P-selectin.

Human citrated plasma caused an inhibition of PTL. It is probable, that the contained sodium citrate may inhibit PTL adhesion by its calcium ion-binding capacity. The flow experiment as dynamic system is in our view absolutely essential for the evaluation of biomaterials for vascular prosthesis, and is in accordance with the international standards. The results of the experiments also suggest that investigations under static and flow conditions are needed to determine the influence of protein adsorption on mixed blood cell populations, for example, on PTL and PMN mixtures/co-cultures in order to achieve a better simulation of the *in vivo* situation.

Introduction

During the last two decades vascular diseases have become more and more important in clinical practice. Since the beginning of the 1950s reconstructive vascular surgery has established its position through the use of artificial prosthetic devices [1].

Since the beginning of the year 2000 approximately 1 000 000 vascular prosthetic devices have been implanted worldwide into the human body. A vessel calibre of the order of 3 mm, as well as venous flow conditions, represents limitations to current vascular prosthetic devices. On the other hand, the demand for an adequate prosthetic device for use in the vascular periphery, for example in coronary revascularization, is a major challenge for biomaterial science.

Biomaterials for implants, including artificial organs [2], induce a specific reaction after implantation in the human body [3]. This reaction depends on the chemical and physico-chemical properties of the material, as well as on the site and type of implantation. The biocompatibility of a biomaterial is not only the absence of direct or indirect cytotoxicity, but also includes the entire field of biosafety and biofunctionality. The International Standard Organization (ISO) in the directive 10993 [4] described the methods for biological evaluation of medical devices as a basis for the assessment of biocompatibility [5, 6].

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In accordance with the recommendations of the ISO, our primary interest focused on the establishment of a reliable *in vitro* panel as a useful screening tool for testing biomaterials for vascular implant devices to evaluate blood/device interactions under reproducible and well-defined flow conditions, as described before [7–9].

Biomaterial-induced thrombosis remains one of the main complications of vascular implant devices, which leads to the loss of function via direct occluding effects as well as many other secondary postthrombotic effects [10]. Today, the thrombogenicity of implant devices is one of the central problems in the analysis of potential biomaterials for vascular devices, but there are uncertainties about how to test appropriately [11].

Platelet (PTL) activation and adhesion are important parameters characterizing the hemocompatibility of biomaterials [11, 12].

They are involved during the early interaction with a biomaterial [13]. During the initial contact between biomaterials and PTLs, PTL-adhesion was described via GP IIb/IIIa receptor activation, whereas the GP Ib/IX-complex was not included in the initial adhesion processes [14]. The first PTL-monolayer on the biomaterial surface appeared to be induced by the GP IIb/IIIa-complex, whereas the secondary layers adhered using GP Ib and von Willebrandt factor [15].

The amount of adherent PTL during the initial contact of an implant device with the blood depends on the blood flow. The wall shear rate as well as the shear stress corresponds with the PTL-deposition on the material surface. The deposition process has a maximum at low shear stress, whereas under high flow conditions the amount of PTL-deposition is reduced [12, 16].

PTL adhesion induces several additional effects. The PTL-deposition at the material surface induces degranulation of the PTL followed by the release of several chemokines, cytokines and growth factors [14, 17, 18] as well as a secondary activation of neutrophils and monocytes [9, 13, 17, 18], which can lead to degradation, and subsequent phagocytosis of the degraded biomaterial.

Implanted materials acquire a layer of host proteins very quickly, well before the arrival of PTL and inflammatory cells [10, 19]. The preadsorbed proteins on the biomaterial/blood interface will modify the adhesion and activation of PTL during the initial contact-phase. Various studies show that PTL adhesion could be improved or inhibited by protein preadsorption [11, 19, 20].

During this time, depending on many conditions, the deposited layer of plasma proteins varies in its composition. Depending on the hydrophilicity, surface charge and chain mobility, proteins are adsorbed to the material surface [21]. By the use of multicomponent protein mixtures the composition of the self-assembled protein layer shows a temporal evolution. This phenomenon, described as the Vroman effect, portrayed the mechanism as an adsorption sequence of albumin, immunoglobins, fibrinogen, fibronectin as well high molecular weight kininogens [22]. The evolution of the composition of the protein layer may thus influence PTL adhesion on the solid–liquid material interface.

The aim of this study was to characterize the influence of preadsorption of different proteins on various polymer surfaces on the adhesion of PTL under static as well as under low- and high-flow conditions to simulate the situation in the arterial and venous peripheral circulation.

For these experiments we used materials with different hydrophilicity to illustrate the effect of material surface properties on the protein-dependent PTL-adhesion.

Materials and methods

PTL-isolation

Freshly obtained citrated blood of a healthy human donor was centrifuged at 200 g for 15 min (highest possible acceleration and deceleration, Varifuge 3.2 RS) at 37 °C. After centrifugation the PTL-rich-plasma (PRP) was removed from the basal leukocyte and erythrocyte phase. The mean concentration of PTL in PRP was 400 000 PTL/µl, counted using a Neubauer chamber as well Casy 1 (Schärfe, Germany) with a capillary diameter of 60 µm, solution 1 : 10 000. For setting the final PTL-concentration of 200 000 PTL/µl the PRP was mixed with PTL-poor-plasma (PPP). PPP was produced by centrifugation of citrated human blood (same donor) at 3000 g (Varifuge 3.2 RS) for 10 min at 37 °C.

The analysis of the rheological properties of the PTL suspension was completed as described previously using [7, 9, 23] a Brookfield rheometer.

Biomaterials

For static experiments, we used 96-well plates made of standard tissue culture polystyrene (TCPS; type 3072, Falcon, London, UK). E. Campbell (Biocompatibles Ltd., London, UK) performed the covalent binding of Phosphatidylcholine (PC).

For the flow experiments, we used TCPS of the same quality as used for static experiments.

Polyethylene (PE) from the concerted action EUROBIOMAT–Hemocompatibility, i.e. medical grade according to the regulations of the USP XXI, US-Federal Standard 209B, and DAB 9, was the standard reference in our experiments [24]. Chamber size-adapted biomaterial samples were washed for 10 min with the help of a strong detergent solution (2% RBS 35; Perstop Analytical, The Netherlands) in distilled water with ultrasound. After sonification, the materials were rinsed in distilled water in order to remove the detergent. To obtain stable conditions in the chamber systems without elastic tension effects, the biomaterial films (PE) were fixed on standard glass slides (Menzel Glas, Germany) using double-faced adhesive tape (Hartmann, Germany). For the flow experiments, the glass and TCPS-slides without any coating were used as ''positive'' controls [23].

Protein preadsorption

For static and dynamic experiments the biomaterials were preadsorbed with fibrinogen (FG), bovine serum albumin (BSA), a mixture of fibrinogen and bovine serum albumin (FG/BSA) or human citrated plasma (CP).

The proteins were used in the following concentrations in phosphate-buffered saline (PBS: 20 mM, pH 7.2):

1. Fibronectin 5 mg/l
2. Fibrinogen (FG: fraction I, type III, Sigma, Germany) 3 g/l
3. Bovine serum albumin (BSA: fraction V, Serva) 43 g/l
4. FG + BSA (FG/BSA) = MIX 3 g/l + 43 g/l
5. Human citrated plasma (CP) = platelet-poor plasma (PPP)

The CP was prepared from fresh citrated human blood extracted by venipuncture technique. The blood was centrifuged for 10 min at 3000 g and 37 °C. The PPP in supernatant was separated from the cell pellet and stored at 4 °C. Fibronectin (Boehringer Mannheim, Germany) was used as ''positive'' control in a concentration of 5 μg/ml.

For static experiments, 250 μl protein solution were added to each well of a 96-well plate for 240 min on a shaking incubator at 37 °C and a shaking frequency of 35 min^{-1}.

After formation of a self-assembling protein layer during this time the protein solutions were removed and the plates were washed twice using PBS. The plates were then immediately used for the adhesion experiments.

For dynamic experiments, the prepared slides were incubated with the protein solutions in Quadriperm-dishes (Coulter, USA). The dishes were filled with 5 ml protein solution for 240 min at 37 °C at a shaking incubator with a shaking frequency of 35 min^{-1}. After preadsorption, the slides were washed twice in PBS and stored for a maximum of 12 h in a humid chamber for use in the flow experiments.

Static experiments

For static experiments, each preadsorbed or control well was allowed to interact with 250 μl of PRP (FACS-experiments) or 150 μl PRP (EIA-experiments). The wells were incubated for 30 min at 37 °C in a shaking incubator. After incubation, the PRP was removed and each well was carefully washed twice with PBS. Then the PTL were fixed using 4% phosphate-buffered formaldehyde (pH 7.2) for 30 min. After fixation the wells were washed with PBS and dried in air. The supernatants were fixed using 1% phosphate-buffered formaldehyde for 10 min for FACS-analysis.

Image analysis

The number of adherent PTL per mm^2 was determined after H&E staining (SOP, Institute of Pathology, Mainz, Germany) using a conventional light microscope Leica DMRB (Leica, Germany) with a JVC CCD-camera (TK-1381, JVC, Japan). All images were digitized using a Hauppauge frame grabber board in a PC with MS Windows 2000 and the imaging software Image Tools 1.27.

FACS analysis

For FACS analysis, 5 μl of the fixed supernatant-samples were diluted in 100 μl PBS/BSA (1% BSA). The samples were incubated for 30 min at 4 °C with the following direct-labeled antibodies:

1. Isotype control: IgG1 κ − FITC (5 μl) + IgG1 κ − PE (5 μl)
2. CD 31 − FITC (20 μl) + CD 41 − PE (15 μl)
3. CD 31 − FITC (20 μl) + CD 62p − PE (15 μl)

The antibodies were from Immunotech (Hamburg, Germany), except for the antibodies for the isotype control, which were supplied by PharMigen (San Diego, USA). The free antibody was eliminated using a washing procedure with 1 ml/sample PBS/BSA (1% BSA) followed by centrifugation and decantation of the supernatant. The cell pellet was resuspended in 500 μl PBS/BSA (1% BSA). For each sample a minimum of 15 000 cells were analyzed. To maximize the effect of PTL-biomaterial contact we used continuous centrifugation at 300 g (Heraeus Labofuge 6000) for 30 min.

Enzyme immunoassay (EIA)

After decantation of the supernatants the wells were washed manually three times using 250 μl PBS (37 °C) per well. Then 150 μl blocking agent (Boehringer, Germany) with H_2O_2 (1%) were used for 30 min (37 °C). Per well 70 μl antibody-solution were used. The CD 62p-antibody concentration was set to 1 : 1000 in blocking agent with 1% BSA. The samples were incubated for 1 h. After incubation the reaction was stopped by decantation followed by a washing procedure with PBS. For CD 62p (biotinylated antibody) streptavidin and biotinylated peroxidase were added to the wells. As detection system 150 μl OPD-substrate solution was used. The development of the plates was made under visual control. The reaction was stopped using 70 μl 3 M HCl per well. The quantitation of optical density was performed using a microplate reader, MK II (Labsystems, Germany) with λ = 492 nm.

Flow chamber system

The flow chamber system consisted of three basic modules: the parallel plate flow chamber, the flow loop and the image analysis system. The flow chamber, consisting of a polycarbonate-based frame, contained two parallel plates, one polycarbonate surface and an opposite frame that consisted of the glass slide with seeded endothelial cells. The exposition area was 637 mm^2 with a chamber height of 100 μm. The rheological properties of this parallel plate flow chamber were analyzed previously [23]. The flow loop (Fig. 1) consisted of a computer-controlled roller pump (Ismatec, Germany) and sterile silicon tubes that were changed after each experiment. The tubes formed a complete circuit with a temperature-controlled reservoir, which consisted of a Teflon tube.

The system temperature was thermostatically controlled at 37 °C. The system pressure was adjusted to 1 cm H_2O. The chamber was observed by the use of an inverse microscope IRBE (Leica, Germany) with a scanning table, which held the chamber in a fixed position. Continuous observation was achieved using a

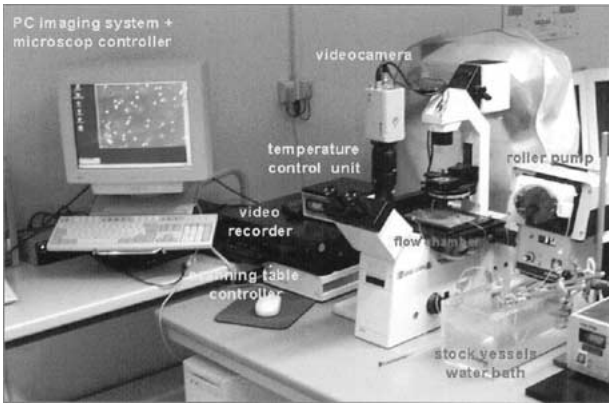

Figure 1 Flow system with parallel plate flow chamber.

three-chip CCD-camera (Sony, Japan). Every minute an image (jpeg-format, 15% compression rate) was obtained using a MicroVideo PCTV-frame grabber board (Pinnacle, Germany) using Webcam32 software version 6.0 (Surveyor, USA).

The morphometrical analysis was performed on a PC Pentium 750 with Windows 2000 using Image Tools 1.27 (University of Texas Health Science Center, San Antonio, USA). Additionally, the whole experiment was recorded using a conventional video recorder (Grundig, Germany). The adhesion process of the PTL was observed over a period of 30 min under arterial and venous flow conditions (shear stress of 0.16 and 1.0 Pa, respectively). The numbers of adherent cells were counted every minute using the IT software. For statistical analysis, the mean number of adherent PTL during the last 5 min of the experiment was used.

Statistics

All statistical evaluations were made using the STATLETS software package version 2.01 from StatPoint LLC (Englewood Cliffs, NJ, USA) that is available on www.statpoint.com. For all data sets, the following statistical tests were made: *F*-test, Student's *t*-test, Mann–Whitney–Wilcoxon test and the Kolmogorov–Smirnov test. For all tests the confidence level was set to 95%.

Results

Static experiments – effect of centrifugation

The number of adherent PTL on native TCPS was 849 PTL/mm^2, whereas on the native PC-surfaces 690 PTL/mm^2 were found. After centrifugation, we found on TCPS 1925 PTL/mm^2 and on the PC-surface 1452 PTL/mm^2 (Fig. 2).

The FACS-analysis showed a high constitutive expression of CD 31 and CD 41 (Fig. 3).

The centrifugation process (300 g) induced a significant ($p < 0.05$) increase of CD 31 and CD 41 expression in supernatant PTLs on PC-coated TCPS, whereas on native TCPS a significant ($p < 0.05$) decrease of CD 31 and CD 41 was induced (Fig. 3).

The results of the FACS analysis of the PTL in the supernatants showed that the PTL activation-marker P-selectin (CD 62p, GMP140) was stimulated by the centrifugation procedure. PTL with PC contact showed a significantly ($p < 0.05$) higher P-selectin expression with and without centrifugation (Fig. 4).

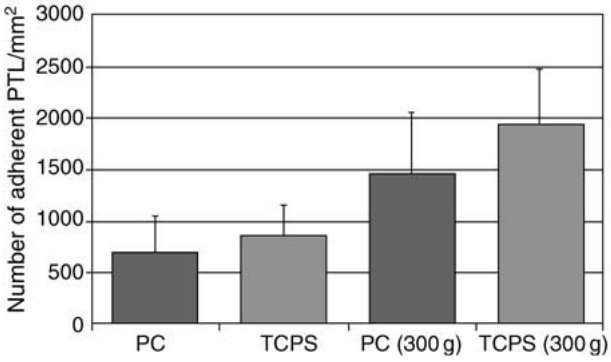

Figure 2 Effect of centrifugation on PTL-adherence on TCPS and PC-coated TCPS.

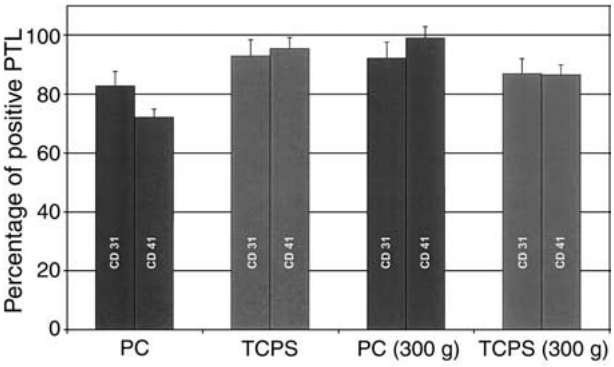

Figure 3 Effect of centrifugation on CD 31 and CD 41-expression on PTL in the supernatants.

Static experiments – effect of protein-preadsorption

During the static experiments, the amount of adherent PTL on native TCPS was measured at 1698 PTL/mm^2 (Fig. 5). The preadsorption of fibronectin induced a significant ($p < 0.05$) increase of adherent PTL to 2111 PTL/mm^2. The highest amount of PTL-adhesion was measured on FG-preadsorbed TCPS with 3901 PTL/mm^2, followed by MIX-preadsorbed TCPS

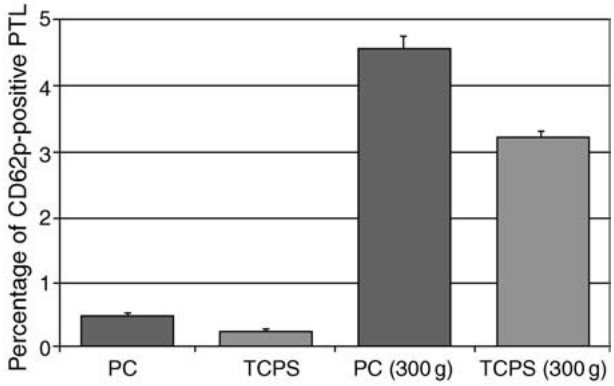

Figure 4 Effect of centrifugation on P-selectin expression on PTL in the supernatants.

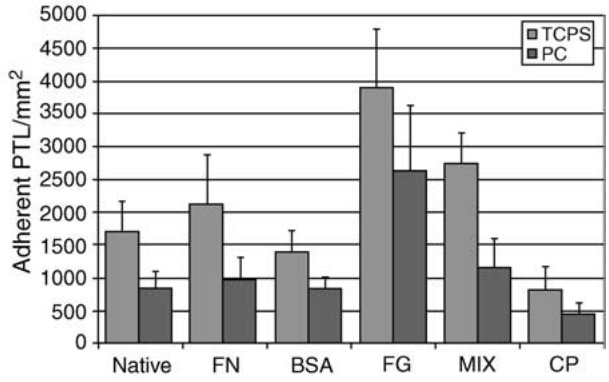

Figure 5 Static experiment: number of adherent PTL on protein preadsorbed TCPS and PC-surfaces.

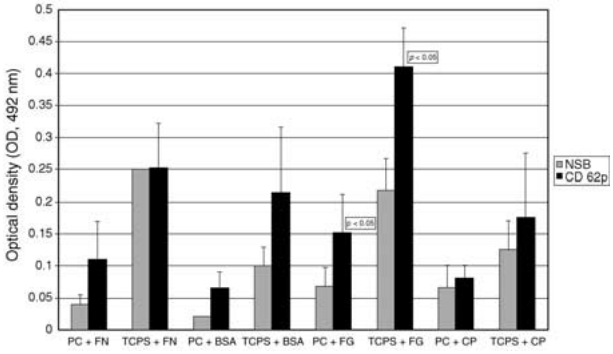

Figure 7 CD 62p-expression on adherent PTL (not normalized for cell number); the red line describes the detection limit for the optical density; significant differences between NSB and specific reaction were set at $p < 0.05$.

(2750 PTL/mm^2), both significantly elevated ($p < 0.05$) in comparison to native TCPS.

BSA induced an insignificant decrease of PTL-adhesion (1397 PTL/mm^2). Only CP induced a significant decrease (837 PTL/mm^2, $p < 0.05$) of PTL-adhesion.

On PC-surfaces (native 837 PTL/mm^2), FN (972 PTL/mm^2) and BSA (845 PTL/mm^2) preadsorption did not lead to any significant ($p > 0.05$) changes. FG (2631 PTL/mm^2) as well MIX-preadsorbed PC (1171 PTL/mm^2) gave a significant ($p < 0.05$) increase of PTL-adhesion. CP-preadsorption on PC showed a significant inhibition (456 PTL/mm^2, $p < 0.05$) of PTL adhesion (Fig. 5).

The FACS-analysis of P-selectin expression of the PTL in the supernatants showed only for the preadsorption with CP a significant ($p < 0.05$) increase. For all other preadsorption protocols a significant influence was not detectable in the supernatant PTL (Fig. 6). On TCPS we found for FN, FG and MIX an insignificant increase of P-selectin expression, whereas on BSA a minimal decrease was measured. For PC-surfaces the preadsorption protocols with FN, BSA, FG and MIX gave an insignificant increase of P-selectin expression.

Static experiments – cell-EIA of CD 62p

The repeated analysis of CD 62p-expression of adherent PTL revealed many technical problems of the EIA, because of the relatively small number of adherent PTL on protein-adsorbed material surfaces. The non-specific binding (NSB: Fig. 7) was relatively high for all assays, frequently being comparable to the specific binding. A significant difference ($p < 0.05$) between NSB and specific binding was only detected for the FG-specimens on TCPS and PC-surfaces. The other analyses gave no reliable results.

Dynamic experiments

The flow experiments of the native materials – glass, TCPS, PE, PC – demonstrated in all cases a relative numerical steady state for PTL adhesion after 25 min of experiment duration.

Under venous conditions ($\tau = 0.16$ Pa) we found the highest PTL-adhesion (Fig. 8) on TCPS (max. 2938 PTL/mm^2, mean 2712 PTL/mm^2). On glass we detected an insignificantly ($p > 0.05$) lower number of PTLs (max. 2470 PTL/mm^2, mean 2305 PTL/mm^2) was detected. PE surfaces induced a significant ($p < 0.05$) reduction of adherent PTL (1000 PTL/mm^2) in comparison to TCPS. The coating of TCPS with PC (188 PTL/mm^2) gave a marked reduction of PTL adhesion in comparison to all other surfaces ($p < 0.05$).

Under arterial conditions ($\tau = 1.0$ Pa) we found the highest amount of adherent PTL was recorded on TCPS (max. 2938 PTL/mm^2, mean 1757 PTL/mm^2). In comparison to TCPS PE (max. 1100, mean 1025 PTL/mm^2)

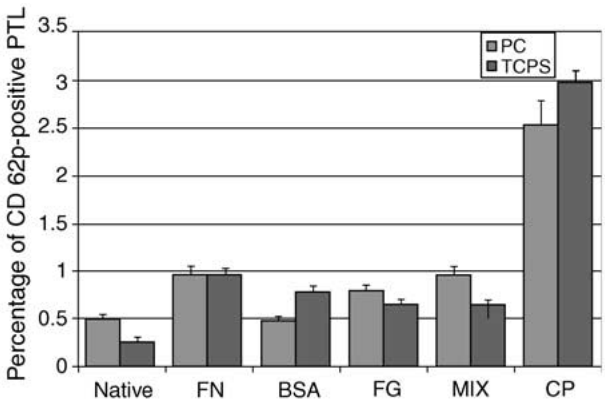

Figure 6 FACS-analysis of the P-selectin-epitope expression with respect to protein preadsorption on TCPS and PC-surfaces.

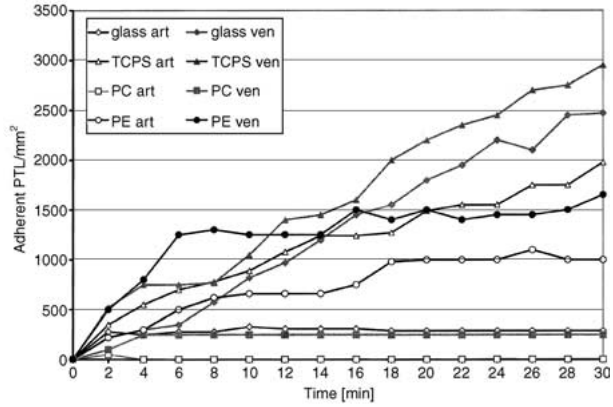

Figure 8 PTL-adhesion on different material surfaces under venous as well as arterial shear stress.

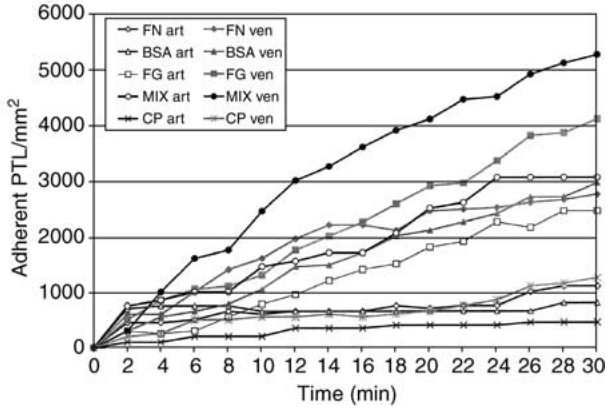

Figure 9 PTL-adhesion on protein-preadsorbed glass under venous and arterial flow conditions.

showed a significant reduction of adherent PTL. On glass we detected a maximum of adhesion, namely with a value of 313 PTL/mm^2 (mean 290 PTL/mm^2). On PC (max. 48 PTL/mm^2, mean 0 PTL/mm^2) no adherent PTL could be detected after 5 min of experiment (Fig. 8).

On protein preadsorbed glass slides under venous shear stress ($\tau = 0.16$ Pa), we measured the highest amount of adherent PTL on MIX-preadsorbed slides (max. 5250 PTL/mm^2, mean 4937 PTL/mm^2). On FG-preadsorbed (max. 4063 PMN/mm^2, mean 3775 PTL/mm^2) slides a significant ($p < 0.05$) reduction of adherent PTL was found in comparison to MIX-preadsorbed slides. BSA (max. 3750 PTL/mm^2, mean 2687 PTL/mm^2) also led to a significantly reduced PTL-adhesion in comparison to MIX and FG. The FN-coating (max. 2688 PTL/mm^2, mean 2627 PTL/mm^2) gave no significant difference in comparison to BSA, but a significant reduction of PTL-adhesion in comparison to MIX and FG-preadsorbed surfaces (Fig. 9). CP-preadsorption (max. 1188 PTL/mm^2, mean 1087 PTL/mm^2) induced the smallest number of adherent PTL in comparison to all other preadsorption protocols ($p < 0.05$). In comparison to untreated glass only CP-preadsorption demonstrated a significant ($p < 0.05$) reduction of PTL-adhesion, whereas (Figs. 8 and 9) BSA gave no significant induction of PTL-adhesion.

Under arterial flow we found a significant reduction of PTL-adherence for all preadsorption protocols in comparison to the venous conditions. The highest amount of adherent PTL under arterial conditions (Fig. 9) was measured on MIX-preadsorbed (max. 3063 PTL/mm^2, mean 3050 PTL/mm^2) glass, followed by FG (max. 2438 PTL/mm^2, mean 2325 PTL/mm^2), FN (max. 1063 PTL/mm^2, mean 987 PTL/mm^2), BSA (max. 800 PTL/mm^2, mean 725 PTL/mm^2) and CP-preadsorbed (max. 438 PTL/mm^2, mean 437 PTL/mm^2) surfaces. All differences, with the exception of the comparison of FN and BSA, were significant ($p < 0.05$).

Discussion

The results of the static experiments demonstrate that on PC-coated TCPS surfaces the number of adherent PTL was significantly reduced in comparison to native TCPS. These results are comparable to the results of Campbell *et al*. [25] and support the theory that covalent PC-coating is a suitable mechanism for inhibition of the prothrombotic effects of potential biomaterials [26]. Ishihara *et al*. [26] discuss that the excellent inhibition of protein adsorption as well as of blood cell adhesion leads to an improvement of the biocompatibility of the materials. This may be supported by our adhesion results, but the results from flow cytometry, especially the results of P-selectin expression, do not support this hypothesis. The clear stimulation of P-selectin expression should be interpreted as a significant activation of PTLs by the PC-coated surface [27], although the proportion of activated PTLs is relatively small. This activation could induce platelet deposition at potential prothrombotic sites. From the point of view of clinical medicine this could increase the risk of thrombotic and embolic events remains unclear. Further experiments must be focused on this effect for an exact risk assessment.

The static experiments for estimation of the influence of protein preadsorption on TCPS and PC-surfaces demonstrated that FG and MIX induce a significant increase of PTL-adhesion. The direct effect of FG on PTL-adhesion is well-known [17, 18] via the GP IIb/IIIa-complex (FG receptor). BSA gave no significant inhibitory effect but the combination of BSA and FG (MIX) demonstrated an inhibition of the prothrombotic effect of FG to approximately 70%. The effect of FN on PTL-adhesion via the GP Ic/IIa is only 54% of the FG effect in our experiment, whereas Grunkemeier *et al*. [20] found a higher adherence of PTL on FN in comparison to FG. In addition to other effects the inhibitory action of CP could involve an effect of the residual citrate in the protein layer on the material surface. Bar *et al*. [28] demonstrated that sodium citrate reduced PTL-adhesion significantly. This effect is comparable to the effect of CP on PMN adhesion [7] on TCPS, which may be induced by the reduction of free calcium ions [29].

The static experiments show that PC induces a significant inhibition of PTL adhesion on the TCPS surface of approximately 50% (range 42.6–67.4%) in comparison to the native TCPS.

The preadsorption of FG and MIX on PC-coated TCPS induces a significant increase of PTL adhesion, but it is a minor effect in comparison to native TCPS. This is a result of the minimal protein adsorption on PC-coated surfaces [26]. Campbell *et al*. [25] described a reduction of FG binding on PC-coated polycarbonate to approximately 10% of the value on native polycarbonate.

The preadsorption of FN and BSA gave no significant effect, again most likely due to minimal protein adsorption on PC. The significant decrease of PTL-adhesion after CP preadsorption is hypothetically induced by the previously described citrate effect [28].

The flow cytometrical analysis of the supernatants of the protein-coated surfaces illustrate that P-selectin was significantly induced by CP. This result must be interpreted as an activation of P-selectin by FG despite the inhibition of PTL-adhesion by sodium citrate. The other preadsorption protocols demonstrate that the supernatant PTL were not affected with respect to P-selectin expression.

The analysis of P-selectin expression of the adherent PTL underlines the technical problems associated with this method. Only the specimens from preadsorbed FG gave reliable results in our test system and indicate that in adhesion assays the use of cell-EIA is problematical, especially for specimens with a relatively small PTL adhesion. Comparable results were achieved for the expression of CD 41b (data not shown). In comparison with the optical analysis the cell-EIA is not suitable for estimation of PTL-adhesion and activation.

The flow experiments show that after a contact time of 25 min a relative steady state of PTL-adhesion was achieved. In accordance with previous studies under venous flow conditions our experiments demonstrate that the amount of adherent PTL differs significantly from arterial flow conditions [7, 9, 16, 22, 23]. For PE, the number of adherent PTL under venous flow was 154% of the arterial value. Native TCPS induced under venous as well under arterial flow the highest number of adherent PTL, whereas PC-coated glass gave almost total inhibition of PTL adhesion. These results confirm the data of the static tests. The ranking order of the other materials (glass, PE) is variable, depending on arterial and venous flow conditions. These data demonstrate that for adequate estimation of the thrombogenic potential of a biomaterial, testing under flow conditions, which are adapted to the planned implantation site, is necessary.

The preadsorption of proteins leads to a modification of PTL-adhesion of the glass surface used. The adsorption of CP induces a significant reduction of PTL-adherence on the material surface. This latter reduction is hypothetically induced by the previously described citrate effect [28]. This observation confirms the result of the static experiments with preadsorbed TCPS. MIX followed by FG induced the highest stimulation of PTL-adherence.

In comparison to our published results with PMN [9] only the adsorption of CP gave a significant reduction of PTL adhesion, whereas all of the protein-adsorption protocols induced a significant reduction of PMN adhesion.

It should be stressed that these results with the individual cell types (PTL or PMN) are not easy to interpret because of the interferences and interactions which take place between PTL and PMN in the *in vivo* situation [17, 30].

Conclusion

Our results clearly show that PTL-adherence on biomaterials is influenced not only by protein preadsorption, but also by flow conditions. The covalent coating of TCPS and glass by PC induces a significant decrease of PTL adhesion but leads to a slight, but nevertheless significant activation of PTL, which was detected by the induction of P-selectin expression using FACS analysis. Methodologically, the visualization of PTL adhesion gave more reliable results for measurement of PTL adhesion than the cell-EIA for P-selectin.

Human citrated plasma caused an inhibition of PTL and PMN-adhesion [9]. It is probable that the contained sodium citrate may inhibit PTL adhesion by its calcium ion-binding capacity. The flow experiment as dynamic system, in our view, is absolutely essential for the evaluation of biomaterials for vascular prosthesis, and is in accordance with the international standards [3–6]. The results of the experiments presented here also suggest that investigations under static and flow conditions are needed to determine the influence of protein adsorption on mixed blood cell populations, for example, on PTL and PMN mixtures/co-cultures in order to achieve a better simulation of the *in vivo* situation.

Acknowledgment

This work was supported by the State Ministry of Science of Rheinland-Pfalz.

References

1. K.-M. MÜLLER and G. DASBACH, in ''The Pathology of Devices'', edited by C. L. Berry (Springer Verlag, 1994).
2. Y. NOSE, *Artif. Organs* **22** (1998) 1003.
3. ISO, ISO 10993 part 1 (1997).
4. ISO, ISO 10993 part 3 (1992).
5. ISO, ISO 10993 part 5 (1999).
6. ISO, ISO 10993 part 4 (1992).
7. M. OTTO, F. BITTINGER, J. KRIEGSMANN and C. J. KIRKPATRICK, *Pathobiology* **69** (2001) 159.
8. M. OTTO, C. L. KLEIN, A. HEROLD, H. KÖHLER, T. VAN KOOTEN and C. J. KIRKPATRICK, *5th World Bionmaterials Congress, Conference Proceedings I* (1996) 705.
9. M. OTTO, B. WAHN and C. J. KIRKPATRICK, *J. Mater. Sci.: Mater. Med.* **14** (2003) 263.
10. W.-J. HU, J. W. EATON and L. TANG, *Blood* **98** (2001) 1231.
11. M. V. SEFTON, A. SAWYER, M. GORBET, J. P. BLACK, E. CHENG, C. GEMMELL and E. POTTINGER-COOPER, *J. Biomed. Mat. Res.* **55** (2001) 447.
12. G. P. A. MICHANETZIS and Y. F. MISSIRLIS, *J. Mater. Sci.: Mater. Med.* **7** (1996) 29.
13. M. OTTO and C. J. KIRKPATRICK, *Biomaterialien* **3** (2002) 133.
14. E. SALZMANN, E. W. MERRILL and K. KENT, in ''Hemostasis and Thrombosis: Basic Principles and Clinical Practice'', edited by R. W. Colman, R. W. Hirsh and V. J. Marder (J. B. Lippincott Company, 1994).
15. R. SHEPPECK, M. BENTZ, C. DICKSON, S. HRIBAR, J. WHITE, J. JANOSKY, S. BERCELLI, H. BORORETZ and P. JOHNSON, *Blood* **78** (1991) 673.
16. H. L. GOLDSMITH and V. T. TURITTO, *Thromb. Haemost.* **55** (1986) 415.
17. P. H. M. KUIJPER, H. I. G. TORRES, J.-W. J. LAMMERS, J. J. SIXMA, L. KOENDERMAN and J. J. ZWAGINGA, *Blood* **89** (1997) 166.
18. J. J. ZWAGINGA, H. I. GALLARDO TORRES, J.-W. J. LAMMERS, J. J. SIXMA, L. KOEDERMAN and P. H. M. KUIJPER, *Arterioscler. Thromb. Vasc. Biol.* **19** (1999) 1549.
19. J. L. BRASH, *J. Biomater. Sci. Polym. Ed.* **11** (2000) 1135.
20. J. M. GRUNKEMEIER, W. B. TSAI, C. D. MCFARLAND and T. A. HORBETT, *Biomaterials* **21** (2000) 2243.
21. H. S. V. DAMME and J. FEIJEN, in ''Modern Aspects of Protein Adsorption on Biomaterials'', edited by Y. F. Missirlis and W. Lemm (Kluwer Academic Publishers, 1991).
22. J. L. BRASH in ''Modern Aspects of Protein Adsorption on Biomaterials'', edited by Y. F. Missirlis and W. Lemm (Kluwer Academic Publishers, 1991).
23. M. OTTO, C. L. KLEIN, H. KÖHLER, M. WAGNER, O. RÖHRIG and C. J. KIRKPATRICK, *J. Mater. Sci.: Mater. Med.* **8** (1997) 119.
24. D. G. CASTNER, B. D. RATNER and D. K. LEACH-SCAMPAVIA, in ''The Reference Materials of the European Communities – Results of Hemocompatibility Tests'', edited by W. Lemm (Kluwer Academic Publishers, 1992).
25. E. J. CAMPBELL, V. O'BYRNE, P. W. STRATFORD, T. A. VICK, M. C. WILES and Y. P. YIANNI, *ASAIO J.* **40** (1994) 853.

26. K. ISHIHARA, *Front. Med. Biol. Eng.* **10** (2000) 83.
27. E. CENNI, D. GRANCHI, E. VERRI, D. CAVEDAGNA, S. GAMBERINI, G. FALSONE and A. PIZZOFERATO, *J. Biomed. Mater. Res.* **36** (1997) 289.
28. J. BAR, A. SCHOENFELD, M. HOD, D. RABINERSON, A. MARMUR, G. J. BROOKS and M. AVIRAM, *Clin. Lab. Haematol.* **18** (1996) 281.
29. J. C. BOS, M. P. C. GROOTEMAN, A. J. VAN HOUTE, M. SCHOORL, J. VAN LIMBEEK and M. J. NUBÉ, *Nephrol. Dial. Transplant* **12** (1997) 1387.
30. B.-G. RHEE and L. V. MCINTIRE, *Chem. Eng. Commun.* **47** (1986) 147.

Received 29 July 2002
and accepted 9 July 2003