The cell function analyser (CFA) – a physiological *in vitro* vascular model and potential alternative to animal experiments

Cornelia B. Reininger
Gefäßzentrum Innenstadt der LMU, D-München

Summary
Cell-vessel wall interactions (adhesion, emigration) and cell-cell cohesion (aggregation) have been assessed primarily in animal experiments. The cell function analyser (CFA) is an in vitro vascular model, in which the three components of Virchow's triad are present in a highly standardised and variable form. The CFA permits visual and quantitative analysis of cellular adhesion, emigration and aggregation under physiologically relevant flow conditions (i.e. to the arteries and to the microcirculation). Although the method does not entail the use of a living animal or of animal tissue, as is true for animal experiments, with the CFA specimen fixation and histomorphological analysis after the experiment is possible. The efficacy of the method for platelet function testing has been verified by numerous clinical studies. The wide variability of test parameters make CFA suitable for in vitro analysis of other cell-vessel-wall-mediated processes, such as inflammation, wound healing and tumour metastasis. We present: 1) a description of the CFA method and underlying hemodynamic principles, 2) a review of clinical and experimental results with platelets and, 3) the first results of convective flow-mediated leukocyte-endothelial interactions. The CFA provides an in vitro alternative to animal experiments, can be classified as a replacement method and possesses an analysis spectrum that will greatly reduce the overall need for the previous.

Keywords: 3R, replacement, flow model, adhesion, emigration, aggregation, platelet, leukocyte, endothelium

Zusammenfassung: Der „Zellfunktionsanalysator" (cell function analyser CFA) – ein physiologisches in vitro Gefäßmodell als mögliche Alternative zu Tierversuchen
Zell-Wand-Interaktionen (Adhäsion, Emigration) und Zell-Zell Kohäsion (Aggregation) im Gefäßinneren wurden bisher überwiegend im Tierversuch analysiert. Der Zellfunktionsanalysator (CFA) ist ein physiologisch-relevantes in vitro Gefäßmodell, das alle drei Komponenten der Virchowschen Trias in hoch standardisierter Form beinhaltet. Mittels CFA ist die visuelle und quantitative Erfassung dieser Zellfunktionen unter für das gesamte Gefäßsystem (Arterien, Venen, Mikrozirkulation) relevanten Strömungsverhältnissen möglich. Obwohl kein tierisches Gewebe benötigt wird, kann auch nach dem Experiment, ebenso wie im Tierversuch, die histomorphologische Untersuchung des Präparates erfolgen. Für die Plättchenfunktionsdiagnostik ist die CFA-Methode bereits klinisch einsatzreif. Aufgrund der einfachen Variierbarkeit der Versuchsparameter ist die Methode geeignet, wichtige Aspekte anderer Zell-Zell- bzw. Zell-Wand mediierter Prozeße, u.a. der Tumorausbreitung und Entzündungsreaktion zu analysieren. Der folgende Artikel beinhaltet: 1) eine Beschreibung der CFA-Methode und entsprechende strömungsdynamische Hintergründe, 2) eine Übersicht über die für Plättchen erzielten klinischen und experimentellen Ergebnisse und 3) die ersten Ergebnisse der konvektiven flußgesteuerten Leukozyten-Endothel-Interaktionen. Der cell function analyser (CFA) bietet eine in vitro Alternative zu Tierversuchen und kann, da kein tierisches Gewebe benötigt wird, zu den Replacement- Methoden gezählt werden. Das Analysespektrum des CFA erlaubt es, den Einsatz von Tieren in großem Umfang zu reduzieren.

1 Introduction

Cell-vessel-wall adhesion with subsequent cellular cohesion (i.e. aggregation) and/or transendothelial migration are basic biological reactions central not only to hemostasis and thrombosis (Fuster et al., 1992), but also to wound healing (Cavenaugh et al., 1998), the inflammatory response, immune recognition (Archelos et al., 1998; Moore et al., 1998) and tumour metastasis (Haung et al., 1997). The mechanisms involved in these multi-step phenomena are thus currently under intense investigation. Cell-cell and cell-vessel wall interactions have been analysed *in vivo* in experiments using a wide range of animals, including dogs (Gold et al., 1991), pigs (Badimon et al., 1989), cows (Naggahata, 1997) and primates (Mousa et al., 1999; Dietsch et al., 1999), and by means of intravital microscopy in rats (Ley et al., 1997), rabbits (Kunkel et al., 1998) and cats (Kubes, 1997). *In vitro* these reactions have been assessed in assays using static wells or rotating systems (Swift et al., 1998) and in wall-parallel flow chambers (Kirchhofer et al., 1993). Aside from ethical reasons to reduce experiments with animals, a major disadvantage of animal experiments lies in the limited ability to manipulate and to vary test parameters and with *in vitro* systems, in the sole application of stasis or wall-parallel flow (O'Brien, 1989).

Fig. 1 a demonstrates the digital subtraction angiography (DSA) of the human mesenteric arteries. As is apparent from

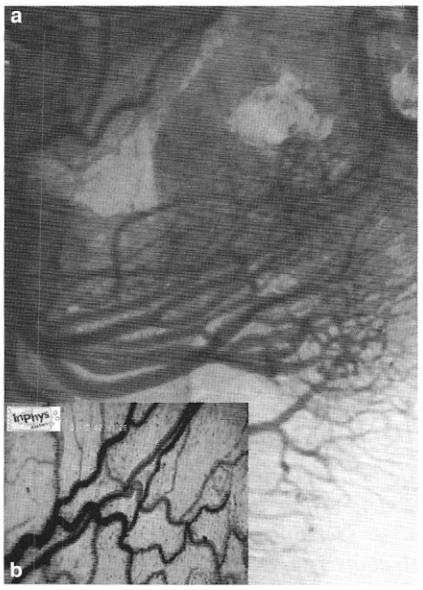

Figure 1a and b: (a) Digital subtraction angiography of the human mesenteric arteries. (b) Intravital microscopy of a rat mesenterium perfused with washed erythrocytes suspended in Ringer's solution (63 x magnification, generously donated by Prof. Holger Schmid-Schönbein). The branchings and curvatures present in both the large and small arteries and in the microcirculation are readily apparent.

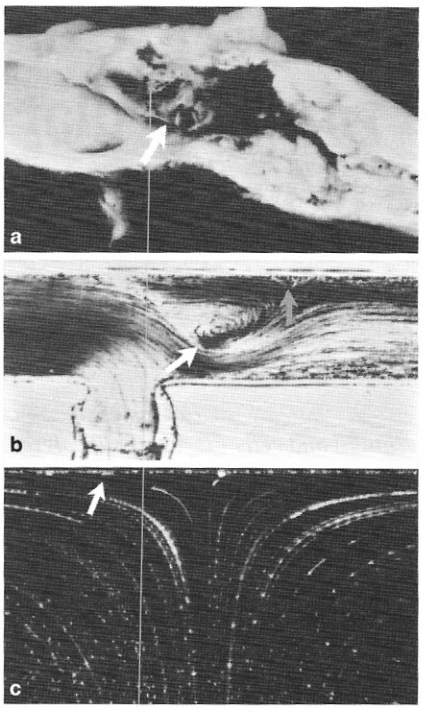

Figure 2a - c: (a) Post-mortem preparation of a coronary artery bifurcation containing a large occlusive thrombus (arrow). (b) Laser light section through a glasmodel of a vessel bifurcation. The large re-circulation zone (white arrow) and region of stagnation point flow (grey arrow) are clearly demonstrated. (c) Laser light section of stagnation point flow in the CFA flow chamber. The platelets (bright spots) and corresponding streamlines (bright streaks) are visualised by means of laser ultramicroscope anemometry (LUA, see text).

the DSA, due to the numerous arterial branchings and curves, almost no longer regions of wall parallel flow exist - either in the large arteries or in the small vessels. The intravital microscopy of a rat mesenterium - seen in Fig. 1 b - verifies that the same is true for the microcirculation. Here, as in the arterial system, only limited regions of wall parallel flow exist; instead non-axial flow, with regions of re-circulation - containing flow separation and reattachment prevails (Sato and Ohshima, 1990; Nimi and Sugihara, 1982; Schmid-Schönbein, 1988).

Numerous clinical and experimental studies have substantiated that, in the arterial system, atheroma and occlusive thrombi develop preferentially in the re-circulation region within a vessel branching or curve and not in the axial regions of wall parallel flow (Goldsmith and Turitto, 1986; Schmid-Schönbein, 1988). Such re-circulation zones act as flow-mediated-reactors, in which long residence times permit accumulation of coagulation factors and polymerisation of fibrin. The area of re-attachment at the distal border of the re-circulation zone is characterised by the presence of stagnation point flow, a flow which provides enhanced convective transport of platelets and other cells to the vessel wall along flow streamlines with components perpendicular to the surface.

To better illustrate the above, Fig. 2 a shows the post mortem preparation of a coronary artery bifurcation containing a large occlusive thrombus (arrow) and Fig. 2 b depicts the flow prevailing in such a branching (visualised by means of laser ultra microscope anemometry; LUA, Kratzer and Kinder, 1986). The large re-circulation zone (Fig. 2 b, white arrow), which directly corresponds to the thrombus region (see Fig. 2 a, arrow), is readily apparent - as is the stagnation point flow (grey arrow). The recognition that the high-particle-surface contact rate accomodated by this flow provides optimal conditions for analysing cell-vessel-wall interactions led to the developement of stagnation point flow adhesio-aggregometry (SPAA, Reininger et al., 1992 and 1996 a). The efficacy of the SPAA method for the analysis of platelet function (i.e. adhesion and aggregation) has been verified on the basis of numerous clinical studies (Reininger et al., 1992, 1994, 1995, 1996 b and c, 1999 b).

We have modified and developed the SPAA method in such a matter as to permit real time analysis of the dynamic interactions of cells other than platelets with a variable biological surface (i.e. endothelium, extra-cellular matrix). This refined system - which we refer to as the cell function analyser (CFA) - is a physiologically relevant *in vitro* vascular model in that all three components of Virchow's triad - the vessel wall, the blood flow and the blood itself - are present in highly standardised form and that, in addition to reproducible analysis of all three, a wide variation thereof is possible. In addition, as no living animal or animal tissue is required, the system can be considered an *in vitro* replacement method for animal experiments.

Generally speaking, the CFA simulates the convective (i.e. stagnation point) flow conditions present within the re-attachement point of a re-circulation zone (see Fig. 2 b, grey arrow), whereby the flow conditions can be adjusted to mimic those prevailing either in the arteries or in the microcirculation. We use dark field microscopy to visualise the cells suspended and superfused in our flow chamber (see Fig. 2 c) and - due to a few technical details-cell-vessel interactions (i.e. rolling, adhesion and aggregation) can be visualised and registered in the original flow field and directly at the wall surface. In the following publication we provide a detailed description of the CFA method, as well as a review of the clinical and experimental results obtained with its prototype for platelets. We also present the preliminary results of leukocyte-endothelial interactions measured with the CFA under flow conditions prevailing in the post-capillary venules.

2 Cohort, materials and methods

2.1 Cohort

The studies described were conducted with the informed consent of all subjects and with the approval of the University ethic committee. All participants (healthy volunteers, congenital *afibrinogenemia*

patients and patients with peripheral arterial disease; PAD) were administered a structured questionnaire based on WHO recommendations for the detection of cardiovascular disease (Rose, 1962) and containing additional questions on smoking habits, concurrent medication and the presence of other illness. For platelet function, the results of four published studies with consecutive collectives of healthy individuals and PAD patients (admitted to our vascular surgery department for reconstruction) are described. The leukocyte-endothelial studies were performed with blood stemming from 9 healthy volunteers.

2.2 Blood sampling and preparation

Fasting blood was drawn with a 20-gauge cannula and minimal or no stasis. The first 10 ml was used for determination of routine laboratory parameters. Blood for the platelet, leukocyte and coagulation studies was then drawn into 10ml syringes containing 1 ml of either 3.8% sodium citrate (platelets) or EDTA (leukocytes). Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 260 g for 10 minutes. After separation of PRP, plasma was prepared by centrifugation of remaining blood at 1000 g for 10 minutes. Plasma fibrinogen concentration was measured according to the Clauss method (Multifibrin, Behring, D-Marburg).

2.3 Cell function analyser (CFA)

The CFA consists of a dark field microscope (Axiophot, Zeiss, Germany) and a stainless steel, removable flow chamber positioned within its object plane (Fig. 3). The CFA allows visualisation and real-time measurement of the convective superfusion of cells suspended in medium (i.e. serum, plasma, buffer) onto a standardised surface (highly cleansed or biologically coated glass coverslip). The suspended cells are conveyed perpendicularly onto the surface at a constant flow rate (30 ml/h for PRP and 15 ml/h the granulocyte suspension; calculated inflow velocity for a volume flow rate of 30 ml/h=5.89 cm/s) and under low shear flow conditions (160 sec-1 or 2.2 dynes/cm^2). The light scattered by adhering cells is photometrically enhanced, converted to volt (photo-multiplier model 476005-9901, Zeiss, Germany) and recorded continuously as a light intensity (growth) curve via a dual-channel recorder (model 8262, Phillips, Germany). At the end of the experiment, the superfused surfaces are removed from the flow chamber and fixed in a 1% glutaraldehyde solution for further phase-contrast (Model IX70, Olympus, Germany) and scanning-electron micro-scopic examination (SEM; JSM, JEOL, Japan). To facilitate storage and computer-assisted analysis of all visual data, the cell-deposition process is photo-(Nikon 301, Japan) and video-documented (Hamatsu, Japan) at regular intervals. A diagram of the experimental set-up is seen in Fig. 4.

Figure 3: Blow-out diagram of the CFA flow chamber with its numerous stainless steel and rubber components, as well as the plexiglass central portion through which the solution to be analysed is perfused. Flow chamber dimensions are as follows: radius of the stagnation plate=1.1 mm, height (h) of the flow chamber=0.6 mm, radius of the inflow tube=0.3 mm.

Morphological definition of cell adhesion and aggregation - based on experiments with platelets. With the CFA measurement and subsequent quantification of both platelet adhesion and aggregation (if present) in the original flow field is possible. Differentiation is based on differences in morphological structure. To elucidate the previous, in Fig. 5 a - d the dark field microphotos taken during the experiment (a and c) and the scanning electron micrographs of the fixed surfaces (b and d) superfused with PRP from a healthy volunteer (a and b) and from a PAD patient (c and d) are seen. The healthy control exhibits non-pathologic adhesion. That is - as demonstrated in the scan image - each individual platelet has direct contact with the wall surface. In the dark field photo (Fig. 5 a) these single platelets are visualised as small light spots aligned next to one another in streaks, that reflect the prevailing flow conditions.

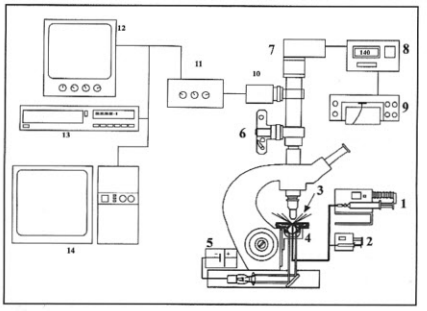

Figure 4: The experimental set-up: (1) perfusion device with siliconised syringe for transport of fluid suspensions at a constant flow rate, (2) micro-perfusion device for continuous addition of test substances during the experiment, (3) stainless steel and plexiglass flow chamber, (4) dark field condensor, (5) light source providing illumination at a constant voltage, (6) data-back camera for automatic photodocumentation, (7) photomultiplier, (8) digital control device for enhancement and conversion of the photometric signal, (9) dual channel recorder, (10) high-sensity video camera, (11) video receiver, (12) video monitor, (13) video recorder (back up system), (14) PC (Hewlett-Packard, USA), with: high resolution video card, real-time documentation system and high resolution Multisync-Monitor (Eizo, Japan).

The patient, on the other hand, exhibits pathologically increased platelet adhesion, depicted in the dark field photo by the enhanced confluency and thickening of the platelet streaks (see Fig. 5 c), and marked spontaneous aggregation, as reflected by the numerous bright spots of aggregated platelets dispersed evenly upon the layer of adherent platelets. Aggregation is defined by the fact that only the bottom of most platelet layer has direct contact with the wall and all other platelets cohere only to one another. This is clearly seen in the electron micrograph (Fig. 5 d).

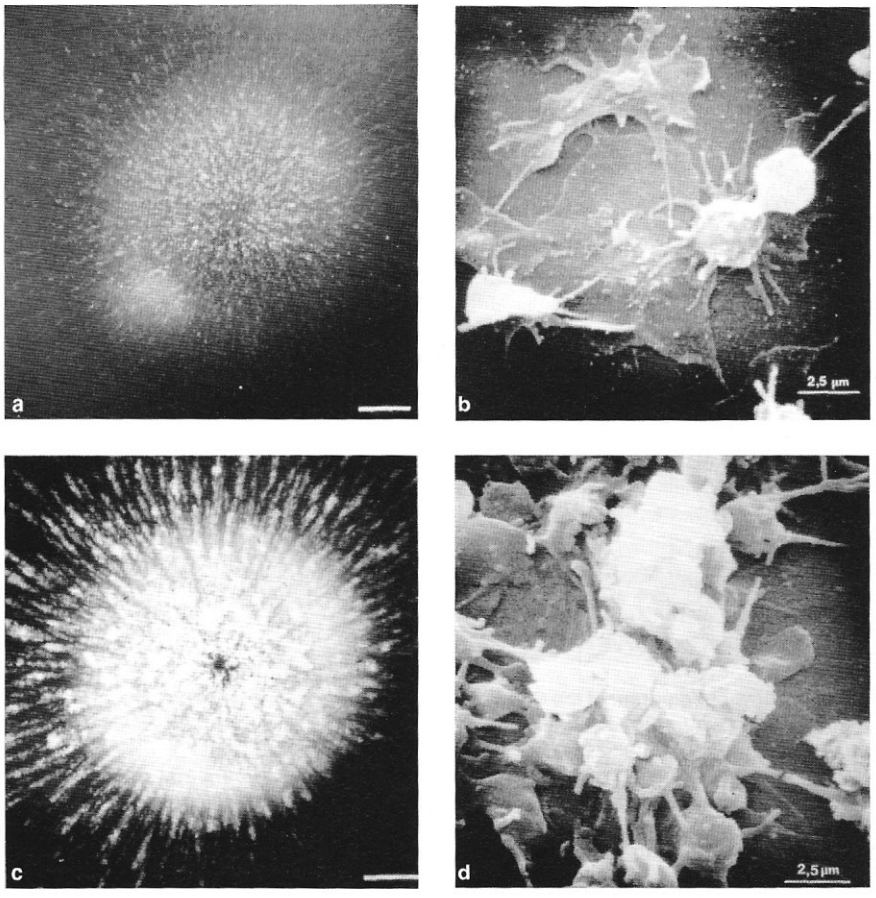

Figure 5a - c: Darkfield-microphotos taken during the experiment (a and c) and scanning electron micrographs (b and d) of the fixed surfaces perfused with platelet rich plasma (PRP) stemming from a healthy individual (a and b) and from a patient with peripheral arterial disease (PAD; c and d). In the dark field photograph the control exhibits the fine streaks of platelets (bright spots) characteristic of non-pathological adhesion (a). In the electron micrograph the contact of each platelet with the perfused surface is verified (b). In the dark field micrograph with PRP from the PAD patient, pathological adhesion is reflected by the increasing confluency of the streaks of adhering platelets and aggregation by the bright irregular spots (aggregates) distributed evenly on the surface (c). Such an aggregate is clearly seen in the electron micrograph (d). The horizontal bars in the darkfield photos represent 0.2 mm.

Quantification of CFA test results. The CFA light intensity curves result from on-line measurement of the light scattered by deposited cells. For subsequent quantification of test results, not only the number, but the morphological structure of these deposits is important. By means of mathematical analysis of these curves using differential equations, which reflect the respective reaction kinetics of the adhesion and aggregation functions, a quantification of real time test data is possible (Reininger, 1999 a). Quantitative results are expressed by two growth curve constants: Kpw (%) reflects platelet adhesion and Kpp (%) aggregation. The mean Kpw values of the patient and control collectives are expressed as mean (standard error of the mean). In contrast to adhesion aggregation is not always present in a CFA experiment. Therefore, for statistical analysis of group means, the odds ratio of occurrence and not the actual magnitude of the numerical value were used.

2.4 Incubation experiments and plasma-platelet exchange studies.

The PRP of 20 PAD patients and 20 healthy volunteers was analysed with the CFA before and after a 5 minute incubation period with: unfractionated heparin (UH, Liquemin, Hoffmann-LaRoche, Switzerland), a low molecular weight heparin (LMWH, Fragmin, Fresenius, Germany), and a heparinoid (Danaparoid Natrium, Organon, Netherlands; Reininger, 1996 b). In an additional 14 patients and 14 controls (Reininger et al., 1999 b) before the experiment, the PRP was incubated for 10 min with each of the two GP IIb/IIIa inhibitors: the non-peptide antagonist Ro 43-8857 (synthesised and generously donated by BASF Pharma, Germany; Alig, 1992) and the monoclonal, chimeric, murine/human antibody, 7E3 (generously donated by Barry S. Coller). Native PRP from the same blood sampling served as control.

In this study as well, prior to the experiment the following aliquots were added to the PRP sample to be perfused: 1. Plasma stemming from 4 PAD patients was added to PRP from 4 healthy volunteers. 2. Plasma from the volunteers was added to a HEPES-buffered suspension of autologous gel-filtered (GFP) platelets stemming from the same blood sampling. 3. Patient plasma from the patients was also added to the volunteer GFP suspensions. Native PRP from the same blood sampling served as control.

2.5 Afibrinogenemic blood

Blood was obtained from a 32-year old female patient with congenital *afibrinogenemia* who had suffered from bleeding episodes since birth. CFA experiments were performed before and immediately after infusion of 2 g human fibrinogen (Haemocomplettan, Behring, Germany).

2.6 Isolation and characterisation of the granulocytes

Granulocytes were isolated according to a standard technique (Hjorth et al., 1981). Briefly blood was centrifuged in 15 ml tubes at 200 x g for 15 min. The PRP was discarded and the tubes re-centrifuged at 1600 x g for 10 min. The PPP was removed and the leukocyte-enriched buffy coat (located above the erythrocyte-containing suspension) collected and diluted with PBS buffer at a ratio of 1:7. The leukocyte suspension was then underlayered with 3 ml of 55% Percoll and centrifuged at 400 x g for 30 min. After centrifugation, the layer containing the granulocytes and red cells was separated and the white cells gently aspirated from the top of the red cell pellet. The residual red cells were removed by short-term, hypotonic lysis. The granulocytes were then washed twice, di-

luted to a concentration of $2x10^6$ cells/ml and suspended in DMEM-HEPES/0.1% HSA until the CFA experiment. Immediately after isolation aliquots of the solution were removed and stained according to May Grünwald to verify the presence of granulocytes, to confirm the viability of the cells by the trypan blue exclusion and for flow cytometric characterisation on the basis of surface CAM expression.

2.7 Isolation and culture of endothelial cells

HUVEC were isolated according to a modification of the method first described by Jaffe et al. (1973). Briefly, the umbilical cord vein was incubated with a PBS-collagenase solution (1mg/ml) at 37° C for 10 min. The harvested cells were cultured in T25 or T75 flasks containing a mixture of endothelial growth and DMEM medium with 20% heat inactivated FCS (1:2; v:v) and a final concentration of 250 U/ml penicillin, 250 µg/ml streptomycin, 50 µg/ml gentamicin, 1.7 µg amphotericin/ml and 30 µg/ml heparin. The HUVEC were cultivated at 37° C in a humidified 5% CO_2-air atmosphere. After 24 h the supernatant was aspirated, the monolayer rinsed with PBS, and fresh culture medium added. The medium was then renewed every 48 h. Confluent monolayers were formed from 3 to 6 days after initial cultivation. Thereafter, the cells were subcultured. Brief exposure to 0.05% trypsin/0.02% EDTA in saline was sufficient to detach the HUVEC from the underlying surface. After washing with PBS, the harvested cells were re-suspended in the above medium and seeded in a T75 flask.

The integrity and purity of the monolayer were confirmed by the typical cobblestone-like appearance of the monolayers and by the characteristic immunohistochemical staining, whereby the purity of culture was substantiated by the absence of a staining pattern characteristic of epithelial cells and/or fibroblasts. The viability of the layer was verified by trypan blue exclusion.

Immediately before the experiment, the HUVEC coated coverslips were placed into the flow chamber and the granulocytes gently portioned in aliquots of 5 ml. A cell aliqout was then diluted with 5 ml of heat inactived, filtrated (0.2µ) HS, transferred into a 10 ml perfusion syringe and convectively superfused onto the HUVEC layer for 10 min (Müller et al., 1999).

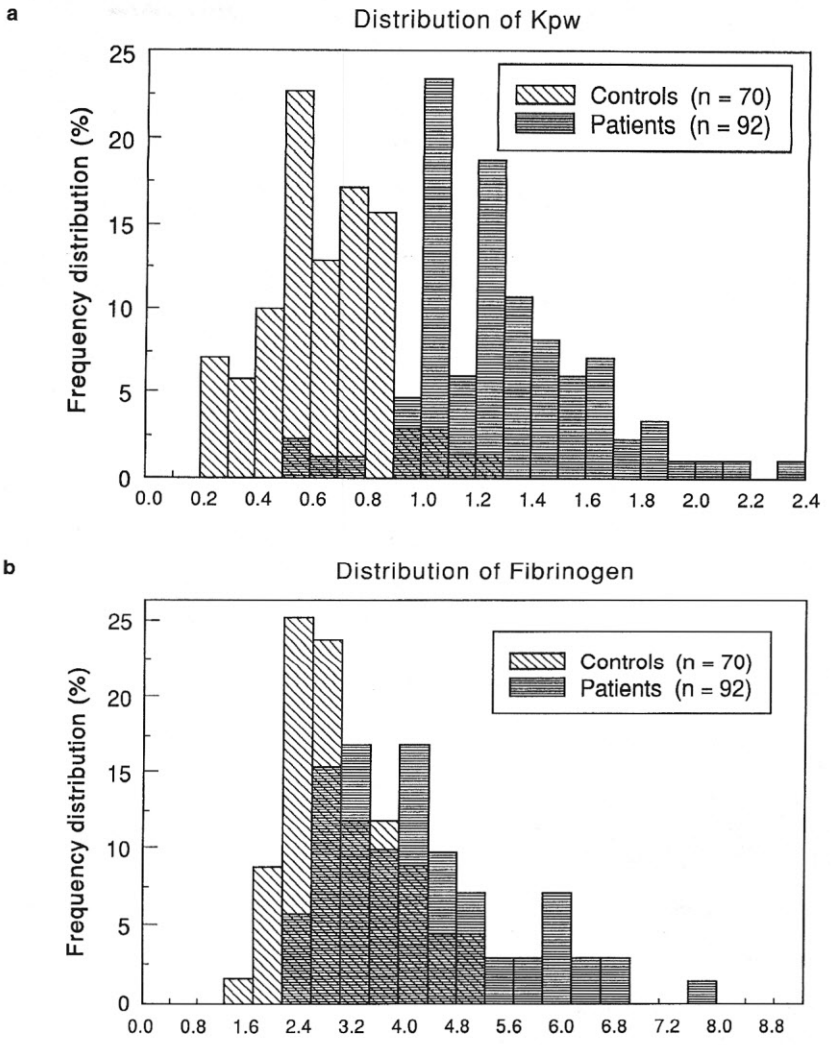

Figure 6a and b: Frequency distribution of platelet adhesion, as expressed by Kpw (a) and that of plasma fibrinogen (b). Please note the minimal overlapping between healthy individuals and PAD patients for platelet adhesion in contrast to plasma fibrinogen.

3 Results

3.1 A review of clinical and experimental results with platelets

CFA-measured platelet function in healthy volunteers and in patients with PAD. In healthy volunteers (n=70), no significant age- or sex-related differences in platelet adhesion were observed. Mean platelet adhesion was higher in smokers (Kpw=0.80% (0.09) *vs* Kpw=0.61% (0.03%)) than in non-smokers. Spontaneous aggregation (Kpp>0) was a rare occurrence in healthy individuals (n=2) and was seen only in smokers. The coefficient of intra-individual variation (CIV) in controls for multiple measurements was 11% (Reininger et al., 1992).

Mean platelet adhesion in PAD patients (n=92, Kpw =1.29% (0.03%)) was over twice as high as that seen in healthy individuals. Aggregation was seen in 61% of the patient collective. No significant differences were seen in smokers *vs* ex-smokers (there were no non-smokers in the PAD collective) or in those taking aspirin compared with aspirin-free patients (Reininger, et al., 1995).

When compared to other parameters of hemostatic activation (i.e. plasma fibrinogen, D-Dimer, Thrombin-Antithrombin-Complex (TAT)), platelet adhesion proved an extremely sensitive and specific parameter in the recognition of manifest PAD. The distribution of CFA-measured platelet adhesion - as expressed by Kpw - in the healthy volunteers compared to the PAD patients is seen in Fig. 6 a and b. The minimal overlapping between the two collec-

tives with respect to adhesion is readily apparent (Fig. 6 a), in particular when compared to the cardio-vascular risk factor plasma fibrinogen (Fig. 6 b). These results corresponded to a sensitivity of 95% and a specificity of 93% for platelet adhesion in the recognition of manifest PAD, when compared to 48% and 84% (respectively) for fibrinogen (Reininger et al., 1996 c).

Effects of heparin on platelet function. The perioperative course of 64 PAD patients following vascular reconstruction was analysed. Post-operatively we noted a marked increase in CFA-measured platelet adhesivity (+0.88% in Kpw) and aggregability (from 25% of the collective up to 75%) - with peak values between the first and fourth days after surgery. This increase in platelet reactivity was paralleled by an increase in plasma fibrinogen concentration and a marked drop in platelet count, down to a minimum of 50% preoperative values on the second post-operative day. As is customary in Germany, during exactly this period, all patients received intravenous unfractionated heparin. We also observed a high incidence of heparin induced thrombocytopenia or HIT antibodies (verified according to the method described by Greinacher et al., 1991) - with and without manifest thrombosis - in one third of the collective (Reininger et al., 1994).

These results prompted us to perform a follow-up study directed particularly towards an analysis of the effects of heparin administration (Reininger et al., 1996 b). The collective included 20 additional PAD patients and 20 healthy individuals. The clinical course in the patients after vascular surgery was analysed. In addition the PRP of healthy individuals and PAD patients (before surgery) was incubated with UH, a LMWH and the above heparinoid. The clinical course in the operated patients and the frequency of HIT antibodies and complications was similar to that observed in the pilot study (see above).

In patients, in the presence of pathologically increased platelet reactivity at baseline, incubation with UH led to a marked and significant increase in both platelet adhesion and aggregation. The effects were less pronounced with the LMWH and minimal with the heparinoid. In the healthy individuals, incubation of PRP led only to minimal and non-significant changes in platelet reactivity, regardless of substance.

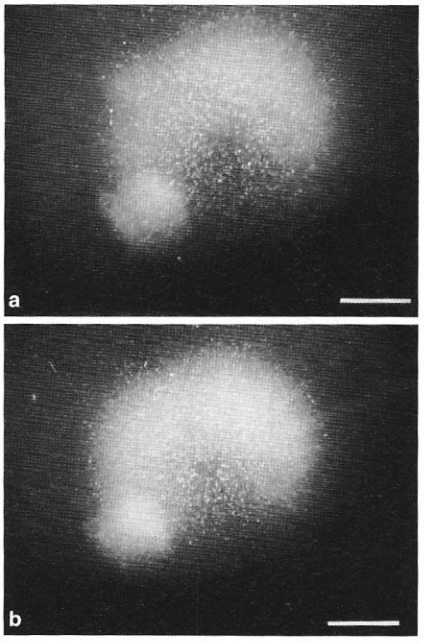

Figure 7a and b: Darkfield microphoto taken during the CFA experiment with PRP from a 46 year old healthy volunteer, before (a) and after (b) incubation with unfractionated (UH). The fine streaks of adhering platelets characteristic of non-pathological adhesion are readily apparent (Kpw=0.37%). The slight increase in adhesion following incubation (+0.11% in Kpw) is seen as a barely visible increase in the confluence of the streaks of single platelets. The horizontal bars represent 0.2 mm.

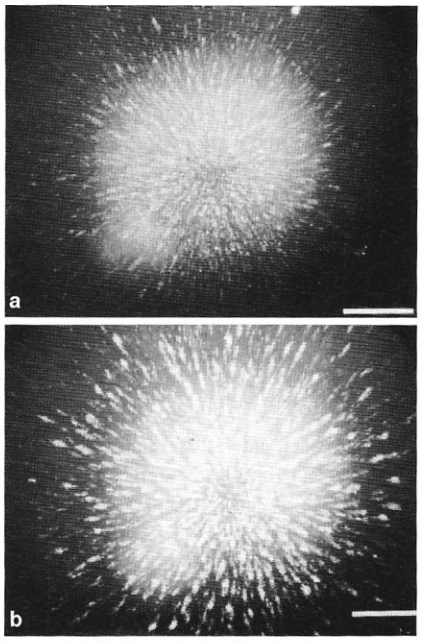

Figure 8a and b: Darkfield microphoto taken during the CFA experiment with PRP from a 46 year old male patient with peripheral arterial disease (PAD) before (a) and after (b) incubation with unfractionated heparin. Note the pathologically increased adhesion (Kpw=1.68%) when compared to the control (Fig. 7 a), as indicated by the enhanced confluence of the platelet streaks. The presence of spontaneous aggregation (Kpp=1.10%) is verified by the white spots of individual aggregates dispersed evenly upon the surface. The marked increase in both functions after incubation is readily apparent (+2.00% in Kpw and +5.00% in Kpp). The horizontal bar represents 0.2 mm.

To elucidate these *in vitro* findings, in Fig. 7 a and b, the dark field photos taken during the experiment with PRP from a healthy volunteer before and after incubation with UH are seen. The minimal change in platelet adhesion before and after incubation with heparin is clearly apparent. In contrast Fig. 8 a and b show the dark field photos with PRP from a PAD patient before and after incubation with heparin. One sees that the already preactivated platelets (Fig. 8 a) become increasingly reactive in the presence of heparin (Fig. 8 b).

3.2 Mechanisms underlying platelet hyperreactivity in PAD patients (below results from Reininger et al., 1999 b)

Afibrinogenic blood: No platelet adhesion was detectable before fibrinogen substitution (Fig. 9 a). After substitution of 2 g fibrinogen platelet adhesion was observed (Fig. 9 b). The Kpw value obtained (0.7%) was within the upper normal range (0.2% - 0.9%). A plasma fibrinogen concentration of 0.16 g/l (one tenth normal value) was measured after substitution.

Incubation experiments: As in all of the above studies, the mean baseline adhesion was twice as high in PAD patients (Kpw=1.16% (0.09%)) when compared to controls (Kpw=0.56% (0.04%)) and aggregation was seen in 10 of the 14 patients compared to non of the controls. Total abolition of platelet adhesion was attained in both patients and controls with 0.5 µM Ro 43-8857 and 10 µ g/ml 7E3. Aggregation, when present, was completely inhibited with 0.1 µM Ro 43-8857 and 1 µg/ml 7E3. Platelet response to inhibition was similar in patients (r=0.927) and controls (r=0.869).

Plasma-platelet exchange studies: Mean adhesion increased three-fold in the presence of patient plasma (Kpw from 0.66% to 1.82%). Patient plasma also caused control platelets to aggregate dur-

ing the CFA experiment (Kpp from 0% to 2.00%). CFA-measured adhesion decreased appreciably after gel-filtration (Kpw from 0.66% to 0.31%). The presence of patient plasma led to an increased ability of the gel-filtered platelets to adhere (Kpw of 0.84%, compared to 0.31%).

3.3 Granulocyte-endothelial interactions: (below results from Müller et al., 1999)

In Fig. 10 a the dark field microphoto during the CFA experiment with non-activated HUVEC is seen. The speckled, light-dark appearance is due to the light refracted by the endothelial nuclei in the dark field environment. Minimal to no rolling and no granulocyte adhesion was seen in experiments with non-activated HUVEC and the corresponding light intensity curve (Fig. 10 b) thus remained flat. After stimulation with TNF, moderate to marked rolling and subsequent firm cell adhesion was regularly observed. The adhering granulocytes are visualised as white, circular structures in the dark field photo (Fig. 11 a, arrows). The corresponding light intensity curve (Fig. 11 b) demonstrates the high amplitude, exponential course typical of cell adhesion. No granulocyte aggregation was seen. Migration of the granulocytes across the HUVEC monolayer was verified upon microscopic analysis of the fixed specimens. Transendothelial migration was time dependent. Fig. 12 a shows an electron micrograph of a fixed superfused surface depicting granulocytes adhering to and emigrating through (arrow) an activated HUVEC surface. Fig. 12 b demonstrates an emigrated granulocyte (arrow) at a higher magnification. The intra- and interassay variance for adhesion was below 10%. A linear dependency was observed between adherent granulocytes and the maximum amplitude of the corresponding light intensity curve.

4 Discussion

4.1 Results with platelets

In patients with PAD atherosclerosis is disseminated and thrombosis risk high (Criqui et al., 1992; Murphy et al., 1998). A primary pro-thrombotic state of the circulation - in which enhanced platelet reactivity plays a major role - has been postulated for these patients (Fowkes et al., 1993). This hemostatic derangement is

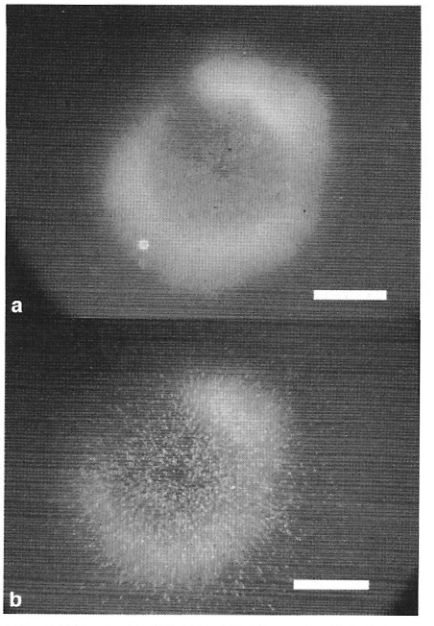

Figure 9a and b: Darkfield microphotos taken during the CFA experiment with PRP from a female patient with hereditary *afibrinogenemia* before (a) and after (b) infusion of 2 g human fibrinogen. Before substitution no platelet adhesion was detectable. After substitution normal platelet adhesion in the CFA system was possible (Kpw=0.70%) as verified by the small bright spots of adhering platelets, aligned next to one another in outwardly radial streaks which correspond to the direction of the flow streamlines.

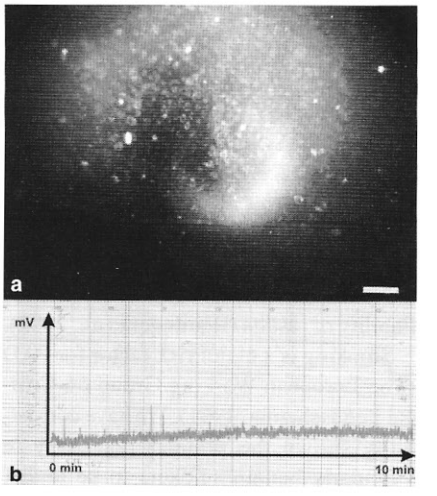

Figure 10a and b: Darkfield microphoto (a) and corresponding light intensity curve (b) following 10 min of superfusion with a PMN suspension onto non-activated HUVEC. The non-uniform speckled, light-dark appearance of the intact monolayer results from the fact that the raised nucle refract light and are thus visible in the dark field environment, whereas the flat cell bodies – that do not scatter light – are not. The flowing, non-adherent PMN are visualised as bright round to oval spheres. Note the almost level course of the light intensity curve. The horizontal bar represents 0.2 mm.

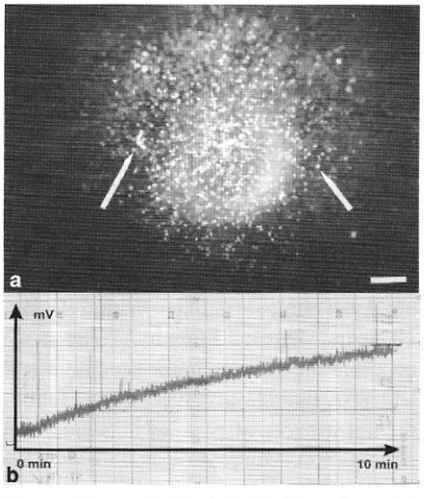

Figure 11a and b: Darkfield microphoto (a) and corresponding light intensity curve (b) following superfusion of PMN onto a with TNF stimulated HUVEC monolayer. The adherent PMN are seen as bright spheres (arrows) dispersed radially outwards from the center of the endothelial monolayer. Note the marked increase in amplitude and the exponential course of the light intensity curve when compared to that obtained in the absence of adhesion. The horizontal bar represents 0.2 mm.

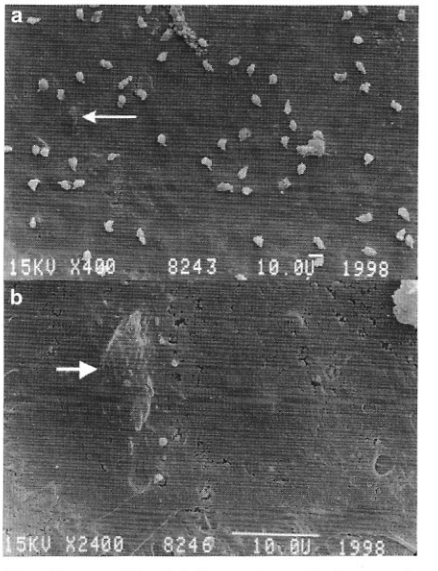

Fig 12 a and b. (a) Scanning electron micrograph of a fixed, superfused, activated HUVEC monolayer coated with adherent and migrating PMN. Note the spiked surfaces and pseudopods of the PMN. The arrow depicts a transmigrated cell. Interruptions in the monolayer were due to critical point drying. In (b) the transmigrated cell is depicted at a higher magnification. The smaller circular structures (arrow) are most likely sedimented human serum albumin.

assumed not only to pre-dispose to acute vascular occlusive events, but to favour disease progression as well (Gonzalez and Kannewurf, 1998). With the CFA we have provided consistent verification of platelet hyperreactivity in PAD patients, whereby platelet adhesion, in particular, proved an extremely sensitive parameter in the recognition of manifest PAD and the minimal CIV of <11% verified the reliability of results (Reininger et al., 1992).

Smoking is a major risk factor for PAD (The European Working Group on Critical Leg Ischemia, 1991). We have consistently shown the platelet reactivity in vascularly benign smokers to be higher than in non-smokers (Reininger et. al., 1992 and 1996 c), whereas in PAD patients, no differences in platelet function were observed in smokers when compared to ex-smokers. In accordance with the results of other authors (Hirsch et al., 1997) our findings indicate that the atherogenic effects of smoking may involve platelet activation, which in some individuals is detectable in the early stages of the disease and that long-term smoking, even when abandoned, may cause irreversible damage to the vasculature (i.e. to the vascular endothelium), which promotes chronic platelet hyperreactivity.

The therapeutic effects of aspirin are limited by its mere inhibition of the thromboxane A_2 pathway, which is not platelet-specific, and by the fact that the potent platelet agonist, thrombin, is aspirin resistant (Coller, 1990). These limitations were reflected in the fact that in the above described studies, CFA-measured platelet adhesion and aggregation were not influenced by aspirin injection. These findings are corroborated by population-based studies, which have reported a beneficial effect of aspirin not in the primary, but only in the secondary prevention of acute cardio-vascular mortality and this to only 30% (Anti-platelet Trialists Collaboration, 1989).

Following peripheral vascular reconstruction, where increased plasma levels of aspirin-resistant thrombin are generated, aspirin combined with a direct or indirect thrombin antagonist such as heparin has been recommended (Jackson and Clagett, 1998). Heparin accelerates thrombin inactivation, but also directly activates platelets and can trigger the autoimmune mediated heparin induced thrombocytopenia (HIT), which can lead to life-threatening arterial and venous thrombosis (Greinacher et al., 1991).

In two consecutive clinical studies (Reininger et al., 1994 and 1996 b) we could verify an *in vivo* hypersensitivity of the platelets of PAD patients to UH, which was indicated by: 1) a marked post-operative increase in platelet reactivity under intravenous heparin therapy, 2) concomitant drop in platelet count of > 50% and, 3) by the high incidence of HIT antibodies, with and without thrombosis. In the latter study, this was confounded by a marked *in vitro* hyperreactivity of PAD platelets to both UH and LMWH.

The above limitations in drug therapy are reflected not only in the high post-operative cardio-vascular mortality rate of this collective, but in the moderate to poor prognosis for limb survival following revascularisation (Pentecost et al., 1994; Gutteridge et al., 1997). In concordance with the findings of other authors (McNamara et al., 1998; Haustein, 1997) our results indicate that heparin-induced immune mechanisms may directly contribute to re-occlusion in PAD patients following re-vascularisation and that - for this reason - application of a direct or indirect thrombin antagonist that does not interact with platelets may improve pre-operative protection in this patient collective.

We have not only provided consistent verification of platelet hyperreactivity in patients with PAD, but have shown them to be aspirin-resistant and to exhibit a paradox response to heparin. In our most recent study with PAD patients (Reininger et al., 1999 b) we have attempted to clarify the mechanisms underlying these regularly observed clinical findings. Within the last 10 years, detailed knowledge has been acquired on the membrane receptors and adhesive ligands involved in the fundamental aspects of platelet adhesion and aggregation. Moreover it is now well appreciated that hemodynamic forces play a major role in determining the modality of platelet responses to vascular injury. Platelets in flowing blood survey the inner lining of the vessel wall without adhering to it under normal conditions, but respond rapidly to the local adhesive surface created by a lesion in the vessel wall. Fibrinogen, although not a native sub-endothelial protein, has been shown to be rapidly immobilised at the site of vascular injury, both by adsorption and/or by deposition of its insoluble derivative (fibrin) (Stanford et al., 1983). Circulating platelets recognise insoluble fibrinogen as the major adhesive substrate where the vascular wall is altered (Ruggeri, 1994). Fibrinogen binding, in turn, is an integral property of the platelet GP IIb/IIIa receptor. Platelets can adhere to immobilised fibrinogen without prior activation and this interaction induces the cytoskeletal re-arrangement and conversion of GP IIb/IIIa to its activated state (Peerschke, 1994). The interaction of the now activated platelets with fluid-phase fibrinogen mediates subsequent platelet aggregation.

Analogous to the above *in vivo* events upon vessel wall injury, our findings with the afibrinogenemic patient, together with the results of an earlier study (Reininger et al., 1996 a) substantiate fibrinogen as being the major adhesive substrate mediating platelets adherence in the CFA system. Here, a plasma fibrinogen concentration of only one tenth the normal level was sufficient to support adhesion. The role of this receptor in also mediating both SPAA-measured adhesion and spontaneous aggregation was also confirmed by the results of the inhibition experiments, in which both functions were completely abolished upon selective receptor blockade.

We noted a marked difference in dose-response to receptor inhibition between adhesion compared to aggregation. Depending upon substrate (i.e. RO 43-8857 *vs* 7E3), a five to ten-fold higher concentration of GP IIb/IIIa inhibitor was required to abolish SPAA-measured adhesion when compared to both primed and to spontaneous aggregation. This higher binding-affinity following platelet adherence may be the result, not only of a net increase in binding sites per fibrinogen molecule (when compared to the fluid-phase protein), but also to the numerous, multivalent contact points provided by the repeating sub-unit structure of fibrinogen in its immobilised form.

The similar response to inhibitory agent in patient platelets when compared to those of healthy individuals indicates their functional normality. The presence of a plasma constituent (i.e. vitronectin from damaged endothelium) that primes these functionally normal platelets *in vivo* is supported by the fact that the addition of

patient plasma markedly increased the reactivity of both native and gel-filtered control platelets. This assumption is further supported by the reports of Fowkes et al., who have verified a variation at the ß-fibrinogen locus, which was significantly associated with an increased risk of peripheral arterial disease and which was not related to an increased fibrinogen concentration alone (Fowkes et al., 1992), and by the *in vivo* and *in vitro* platelet hypersensitivity to heparin we (Reininger et al., 1994 and 1996 b) and others (Hach-Wunderle et al., 1997) have seen in these patients. Heparin is not only reactive with platelets, but with most plasma proteins as well, some of which it precipitates (Salzman et al., 1980). Thus a structurally variant fibrinogen (McDanaugh et al., 1994), or some other reactive protein, may contribute to the *in vivo* priming of platelets circulating within the damaged vasculature of these patients, which (in addition to increasing their baseline activity) leads an *in vitro* (and *in vivo*) increase their reactivity in the presence of heparin.

On the basis of these findings we conclude that: 1) the CFA measures and quantifies platelet interactions with both fluid-phase (aggregation) and immobilised (adhesion) fibrinogen, 2) these reactions are mediated by the GP IIb/IIIa receptor complex, 3) the binding affinity, metabolic pathways and signal transduction underlying platelet adhesion differ from those involved in aggregation (possibly reflecting their varying roles in hemostasis), 4) the functionally normal platelets of patients with PAD are primed *in vivo* by a circulating plasma constituent, which leads to enhanced recruitment of activated GP IIb/IIIa onto the platelet surface and, thereby, to an overall increase in reactivity.

4.2 Granulocyte-endothelial interactions

Leukocyte rolling with subsequent adhesion and endothelial migration are essential events in thrombogenesis and the inflammatory response, a response central not only to infection, but to ischemia/reperfusion injury and carcinogenesis. The mechanisms involved in this multi-step phenomenom are thus under intense investigation. Aside from the knock-out mouse, which is valuable for clarification on the molecular level, leukocyte- and cancer cell-endothelial interactions (i.e. adhesion and transendothelial migration) have been analysed primarily by means of intravital microscopy. The major disadvantages of this method lie in the limited ability to manipulate and vary test parameters as well as in differentiating cell type (i.e. granulocytes *vs* monocytes). In addition, although this method provides a global overview of the flow in the entire microcirculatory network, a quantitative analysis of the complex flow phenomena is not possible with simple mathematical techniques. *In vitro* flow chambers simulate the flow in particular geometrical regions within this vascular in a simplified, but highly standardised and thus quantitatively assessable form. Hereby a parallel plate chamber simulates the flow occurring within the straight regions of the vasculatur, permitting analysis of shearing effects and diffusion-mediated (i.e. passive) adhesive processes. With a stagnation point system, such as the CFA, one simulates the convective flow occurring with the branchings and curvatures of the vessels. A system of this type is optimal for assessment of adhesion-mediated processes. The previous assumption was supported by our present data with granulocytes and endothelium, in which visual and quantitative assessment of rolling, adhesion and subsequent transendothelial migration - that is with the major components of the granulocyte interaction with inflamed endothelium - was possible. The minimal intra- and intertest variance verified the reproducibility of test data and the ability to measure transendothelial migration will considerably increase the method's spectrum of application. It should be mentioned at this point that intravital microscopy and the CFA are complimentary and not competitory systems and that they are not directly comparable with one another. With the former a qualitative observation of flow patterns, cellular behaviour patterns and shunting effects is possible, whereas the latter provides standardised and quantitative measurement of cellular behaviour under limited and highly defined flow conditions.

5 Conclusion

The CFA as an alternative to animal experiments. CFA is a highly standardised *in vitro* system, which permits visual and quantitative analysis of cellular rolling, adhesion and subsequent emigration, as well as aggregation (if present) under physiologically relevant flow conditions. The method can reduce the need for animal experiments for numerous reasons:

5.1 The results of animal experiments - due to numerous and variable confounding effects - are not always directly applicable to humans. In the CFA only human tissue and cells are used, thus avoiding this problem of restricted applicability. On the other hand - as is the case in experiments with animals - specimen fixation and histomorpho-logical analysis is possible. Due to the complete absence of animal tissue in the CFA experiment, the method can be considered a classic *replacement* method.

5.2 A definite advantage when compared intravital microscopy, the primary *in vitro* system used for analysing the above cellular functions is the ability to manipulate test parameters. In contrast to the animal model, all three components of Virchow's triad (Virchow, 1856) - the vessel wall, the blood flow and the blood itself - are present in highly standardised form and that, in addition to reproducible analysis of all three, a wide variation thereof is possible. This wide variability of test parameters (i.e. in flow conditions used, in composition of superfused medium and in the biological surface) make the CFA suitable for *in vitro* analysis of other adhesion and transendothelial emigration processes, such as ischemia/re-perfusion injury, tumour metastasis and wound healing.

5.3 The CFA is not only suitable for assessing platelet-mediated thrombosis risk, but for direct *in vitro* and *ex vivo* analysis of the effectivity of the newly developed antithrombotic substances (i.e. GPIIa/IIIb receptor blockers, direct and indirect thrombin inhibitors). Up to present the testing of such substances has been performed largely in animal models.

CFA data is thus useful not only in elucidating important aspects of athero- and thrombogenesis as well as the leukocyte response to infection, but in evaluating clinically beneficial interventions thereof and thus in reducing the need for - for this purpose - previously required animal experiments.

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Correspondence to
Cornelia B. Reininger, M. D., Ph. D.
Gefäßzentrum Innenstadt der LMU München
Pettenkoferstr. 8a
D-80336 München
Tel.: + 49 89 5160 3601
Fax: + 49 89 5160 4443