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PLATELET ADHERENCE TO SUBENDOTHELIUM OF HUMAN ARTERIES IN PULSATILE AND STEADY FLOW

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ABSTRACT

Platelet adherence to human artery subendothelium was investigated in pulsatile and steady flow in an annular perfusion chamber. The platelet adhesion was larger in steady flow than in pulsatile flow. The ratio of contact platelets to spread platelets was higher in pulsatile flow. The number of adherent platelets increased at increasing platelet number, red cell concentration, perfusion time, and flow velocity in both systems. The difference in platelet adherence in the two flow systems decreased at increasing adhesion, indicating that saturation occurred. The experiments indicate that for comparative studies of platelet adhesion in steady flow at a physiological red cell concentration and a shear rate as observed in small arteries, it is preferable to apply sub-normal platelet counts and perfusion times shorter than 5 min.

INTRODUCTION

Adherence of blood platelets to rabbit subendothelium has been investigated in an in vitro perfusion model (1, 2, 3, 4). In this perfusion model rabbit subendothelium is exposed to citrated whole blood or native blood (5) under controlled conditions, such as composition of perfusate, flow rate, and duration. The vessel wall is exposed to a laminar flow (6). We have applied this perfusion model to human components (7, 8). In oder to improve the flow conditions and to determine the effect of flow pulsatility on the platelet adhesion we have developed a perfusion system with steady flow. The platelet adherence obtained in this perfusion system was compared to the adherence in pulsatile flow under otherwise similar conditions. Optimal conditions for comparative studies of platelet adhesion were derived from these experiments. Also, we compared the platelet recovery and the degree of haemolysis in these perfusion systems.

MATERIALS AND METHODS

Human post mortem arteries. Human renal arteries were obtained about 12 hours after death. The arteries were stored at 4°C in 0.2 M Tris-HCl buffer (pH=7.4) containing 200 U penicilline G (Nogepha, Holland)/ml and 0.2 mg streptomycin (Gist-Brocades, Holland)/ml, and applied in the experimental procedures within 3 days. Arteries with macroscopic plaque formation and atherosclerosis were discarded. Only arteries after the first bifurcation were utilized as their luminal diameter matched the rod diameter of the perfusion chamber. The vessel segments were wiped with a bud probe (a plastic rod, diameter of 2 mm, with a knob at the end with a similar diameter) and everted as described by Baumgartner (9). The everted artery was mounted on the middle of the rod and exposed to air for 3 min and rinsed well with 0.2 M Tris-HCl buffer (pH=7.4) before exposure to the perfusate. Wiping of the vessel lumen with the bud probe, stretching of cells and tissue during the everting procedure, and exposure to air, completely removed the endothelial cell layer (10).

Perfusion systems. Two perfusion systems were used, one with pulsatile flow and one with steady flow. The components of these systems, a peristaltic non-occlusive pump with three rollers (VRE 200, Verder, West-Germany), a perfusion chamber according to Baumgartners original chamber (9, 11), silastic tubings and containers for perfusates were identical. The rod of the perfusion chamber had a minor modification compared to the original model. The solid portion of the rod fitting the outer cylinder of the perfusion chamber consisted of two rubber bands instead of one. In this way the position of the rod was fixed.

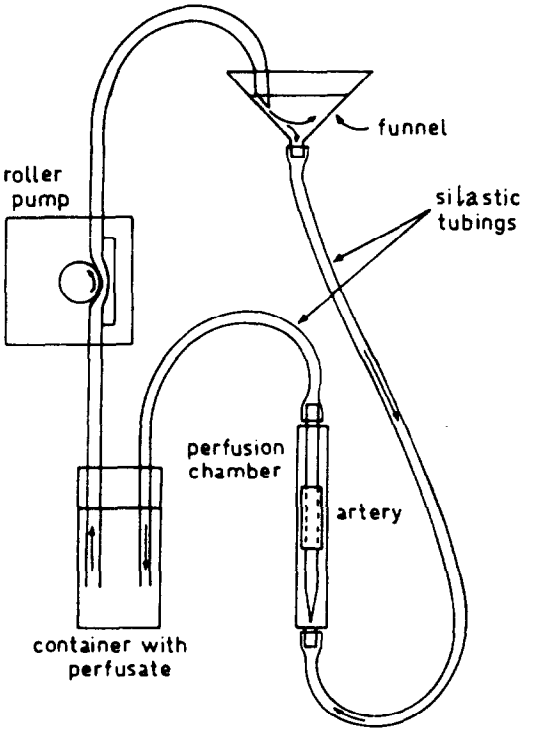

Fig. 1

Steady flow was produced by means of gravity.

Steady flow was produced by means of gravity (Fig. 1). In this system a plastic funnel was included, and the perfusate volumes were in the range of 16 to 20 ml. The length of the silastic tubings were 88 cm. Approximately 2.5 ml of the perfusate was at all times in the funnel during the perfusions, and a fixed height between the funnel and the outflow of the perfusion chamber were maintained. The flow rate was checked at a perfusate temperature of 37°C. When the red cell concentration or the flow rate was changed, the height difference between the funnel and the perfusion chamber was adjusted to the propriate level.

Pulsatile flow was obtained in the same system (Fig. 1), however without the funnel. The volume of the perfusates were in the range of 8 to 12 ml, and the length of the silastic tubings were 77 cm. In some experiments with pulsatile flow, the funnel was placed between the perfusion chamber and the blood container. In these experiments the perfusion volume and the length of the silastic tubings were similar to that of the steady flow perfusion system.

The perfusions were performed at 37,0°C. In the perfusion system with pulsatile flow, with or without the funnel, the temperature remained constant at the outflow of the perfusion chamber at all perfusion times applied. In the perfusion system with steady flow a slight decrease of temperature was detected. During the perfusion, the temperature at the outflow of the perfusion chamber decreased to 36.9°C in 2 min and 36.5°C in 5 min.

Perfusates. 9 parts of blood from healthy donors was anticoagulated with 1 part of citrate, which resulted in a final plasma concentration of about 19 mM citrate. Labelling, aspirin treatment, and washing of platelets and red cells were performed as described (7,8). Reconstitution of the blood components (8) was carried out with different red cell and platelet concentrations, as indicated in the legends. The platelet concentration in the perfusates is given as number of platelets per liter plasma in the absence of red cells. In some perfusates, normal plasma was replaced by platelet poor plasma from a patient severely affected with Von Willebrand disease (7), but the platelets and the red cells were from normal individuals. The perfusates were recirculated for 1 to 20 min, after 5 min preincubation at 37°C.

Determination of subendothelial surface and radioactive registration. After perfusion, the perfusion system was rinsed with 40 ml of 0.2 M Tris-HCl buffer (pH=7.4). Both ends of the vessel segment (about 0.3 cm^2) were cut off, and the remaining subendothelial surface on the rod was calculated from the average diameter of the rods with human arteries (0.394 cm), and the length of the exposed subendothelial surface. The vessel segment was transferred to a gamma counter (trigamma 600, Baird Atomic Inc., Bedford, Mass. USA) for couting of ^{51}Cr.

Determination of number of adherent platelets per cm^2 subendothelium. After the final resuspension of the washed platelets, the radioactivity was measured. The activity was expressed as cpm per 1000 platelets. This specific platelet activity was used to calculate the number of adherent platelets per cm^2 subendothelium after registration of the ^{51}Cr-count associated with the arterial segments. Aspirin inhibits platelet aggregation when four times washed platelets are perfused over human subendothelium (7). Therefore the registrated ^{51}Cr-count represents adherent platelets.

Preparation of arterial segments for light microscopical morphometric evaluation. The vessel segments were fixed in 2.5% glutaraldehyde and 2.0% osmiumtetroxide, and embedded in Epon (9) immediately after perfusion. Epon sections of 1.0 µm were cut with glass knives on an ultratome (8800, LKB, Bromma, Sweden), and stained with toluidine blue and basic fuchsin (12).

Morphometric evaluation. The morphometric evaluation was carried

out at 10 µm intervals on the vascular surface using a Leitz light microscope with a calibrated scale (1). The intersection points were divided according to Baumgartner et al (2) in the following qualitatively different types :(a) naked, subendothelial surface devoid of the platelets, (b) contact platelets, platelets present on the subendothelium,but not spread on the subendothelium, (c) spread platelets, platelets spread on the subendothelium, and (d) adherent platelets, the sum of contact and spread platelets. Findings obtained from the cross-sections (about 1100 evaluations per cross section) were expressed in percentage of total intersection points. The platelet coverage was calculated from these percentages after correction for the systematic error due to the finite thickness of the section (13, 14).

Physical parameters of the perfusion systems. Variations at 37°C in the flow velocity and the corresponding flow amplitudes were measured at flow rates of 60, 135, 165 and 210 ml/min. A cannulated probe (Nytroton, Sweden) was mounted at the outflow of the perfusion chamber. This flow probe detected the flow velocities and the flow amplitudes. A flow meter (developed by Dept. of Electronics, Univ. Hosp. Utrecht, Holland) registrated these parameters and they were simultaneously recorded (recorder: Gold-Brush 480, Gould Inc., Ohio, USA). The response time was low enough to measure frequencies of 100 sec^{-1}. Vessel wall shear rate, shear stress and Reynolds number were calculated according to Bird's formulas for laminar flow in an annulus (14). It should be emphasized that whole blood does not behave as a pure Newtonian liquid, due to the presence of the blood cells (16, 17, 18).
At the edges of the artery-segment flow turbulence occurred. The length of the turbulent area is about 0.03 Rd, where R is Reynolds number and d is the width of the annulus (19). The turbulent area was calculated to be about 0.25 cm long at 135 ml/min in steady flow.

Tests applied on blood cells after perfusion. Haemolysis was determined after perfusion for 5 min in pulsatile and steady flow by means of plasma haemoglobin absorption at 567 nm (20). As controls, non-perfused perfusates were used. Recovery of washed platelets was determined after perfusion for 5 min by means of a thrombocounter (Coulter Electronics Ltd., England).

RESULTS

1. Flow parameters

Table I gives the flow parameters.

Table I. Flow parameters.[a)]

flow rate[b] (ml/min)	amplitude[b] (ml/min)	frequency[b] (cycles/sec)	flow velocity (mm/sec)	shear rate[c] (sec^{-1})	shear stress[c] (g/cm·sec)	Reynolds number
60	130	3.9	59	357	18	47
135	160	8.4	133	804	31	80
165	190	9.1	161	981	38	98
210	180	11.0	206	1246	48	124

a) In pulsatile flow these parameters represent average values.

b) Flow parameters experimentally determined, mean of two determinations.

c) Flow parameters theoretically calculated at the vessel wall (15).

Fig. 2 presents flow-measurements of the two perfusion systems at a flow rate of 135 ml/min.

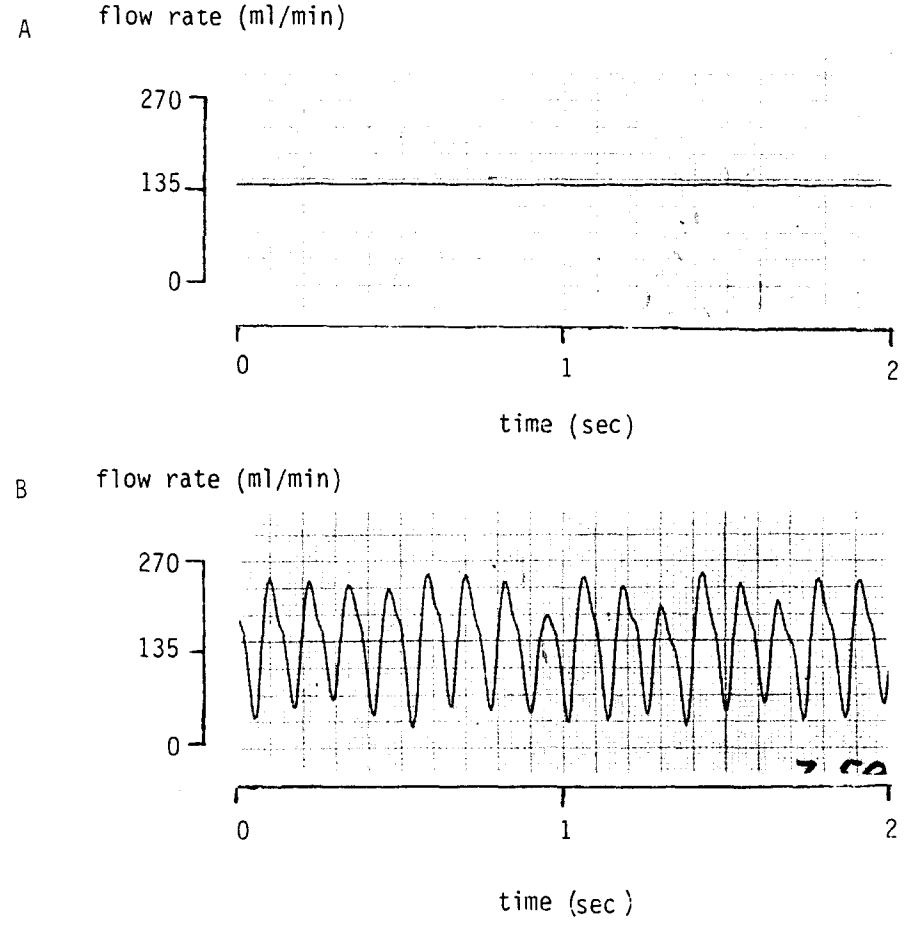

Fig. 2: Pulsatile and steady flow at an (average) flow rate of 135 ml/min.
A). Constant flow system based on gravity indirectly driven by a non-occlusive roller pump.
B). Pulsatile flow system directly driven by a non-occlusive roller pump.

In the pulsatile flow system, back-flow was only detected at the lowest applied flow rate, 60 ml/min. In the perfusion model based on gravity indirectly driven by the non-occlusive roller pump, a constant flow was measured at all flow rates.

2. Distribution of ^{51}Cr-count on the length of the subendothelium.
The platelet adhesion along the arterial segment was investigated with about 1.5 cm long vessel segments. After perfusion, the arterial segments were cut in six orientated transverse strips, approximately 0.25 cm^2 each, and ^{51}Cr was measured. On both ends of the vessel segment, more adherent

platelets were found than on the four middle pieces which on the average had a similar distribution of platelets. The total number of platelets calculated per vessel segment was set to 100% and their distribution on the six pieces is shown in Fig. 3. The distribution of adherent platelets was similar in the two perfusion systems.

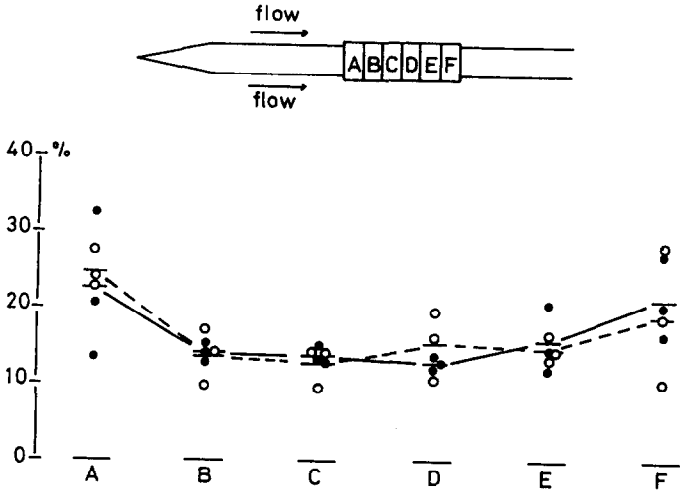

Fig. 3

Dependence of platelet adhesion on the length of the subendothelium. ——● steady flow, - - - o pulsatile flow. The perfusion time was 5 min, the platelet count 1.9 x 10^{11}/l plasma, the (average) flow rate was 135 ml/min, and the red cell concentration was 40%.

3. Platelet adhesion and platelet count.
The influence of the platelet count on the platelet adhesion was investigated with platelet counts varying from 0.25 to 3.00 x 10^{11}/l plasma, Fig. 4A. An increase in platelet count resulted in increased platelet adhesion, but at platelet counts above 2.50 x 10^{11}/l plasma the platelet adhesion was similar in both flow systems. In the pulsatile flow a relatively steep increase of platelet adhesion was observed at platelet counts between 2.00 and 2.50 x 10^{11}/l plasma, which was not observed in steady flow.

4. Platelet adhesion and red cell concentration.
Platelet adhesion was quantitated at red cell concentrations of 20 and 40%, Fig. 4 B.
The red cell concentration had a striking effect on the platelet adhesion in both flow systems. In steady flow about six-fold more adherent platelets were found at a red cell concentration of 40% than in experiments without red cells. In pulsatile flow the difference was about ten-fold.

5. Platelet adhesion and perfusion time.
Platelet adhesion after 1, 2, 3, 4 and 5 min was investigated. Platelet adhesion increased with increasing perfusion time in both flow models (Fig. 5, A). For these conditions the platelet adherence was about twice

as high in steady flow as in pulsatile flow.

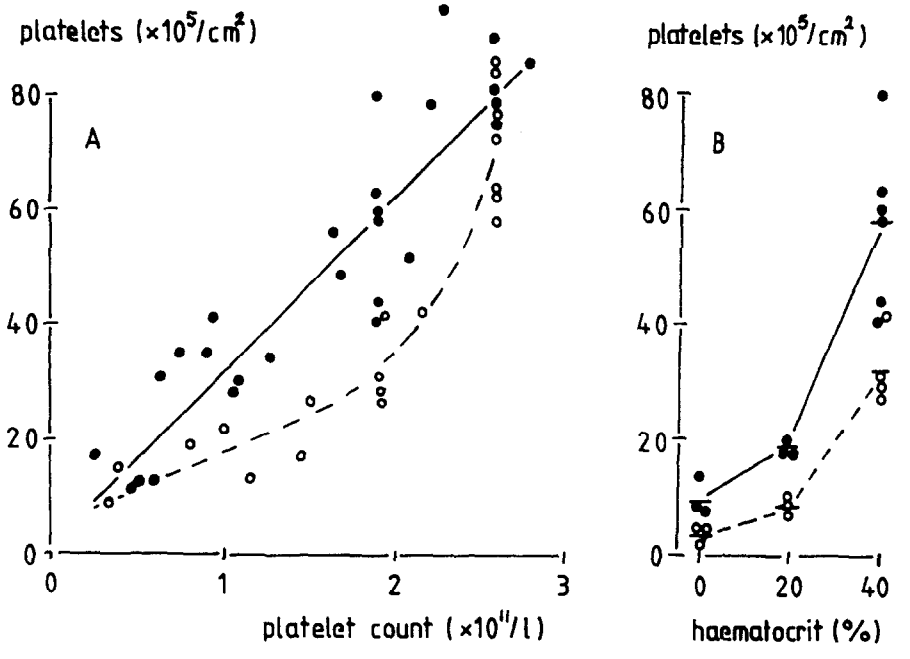

Fig. 4, A and B

——— • steady flow, - - - o pulsatile flow.

A. Dependence of platelet adhesion on the platelet number. The perfusion time was 5 min, the (average) flow rate 135 ml/min, and the red cell concentration was 40%.

B. Dependence of platelet adhesion on the red cell concentration. The perfusion time was 5 min, the platelet number 1.9 x 10^{11}/l plasma, and the (average) flow rate was 135 ml/min.

6. Platelet adhesion and flow rate.
The influence of the flow rate on platelet adhesion was investigated at flow rates of 59, 133, 161, and 206 mm/sec (average flow rates in pulsatile flow) (Fig. 5, B). Increasing flow rate resulted in increased platelet adherence , but more adherent platelets were observed in steady flow as compared to that in pulsatile flow. However, at the two highest flow rates tested, the difference in platelet adhesion between the flow systems was slightly less.

7. The influence of the funnel on the platelet adherence in pulsatile flow.
To test whether the funnel influenced the platelet adherence, the funnel was positioned between the perfusion chamber and the blood container. Thus pulsatile flow was sustained. Perfusions lasting for 5 min were performed with a red cell concentration of 40% and 1.9 x 10^{11}/l plasma.

The average flow rate was 135 ml/min and the perfusion volumes were in the range of 16 to 20 ml.

The platelet adhesion (mean: 26.0 (± 2.7) x 10^5 platelets/cm^2, n=4) was slightly lower than without the funnel (31.3 (± 3.1) x 10^5 platelets/cm^2, n=4).

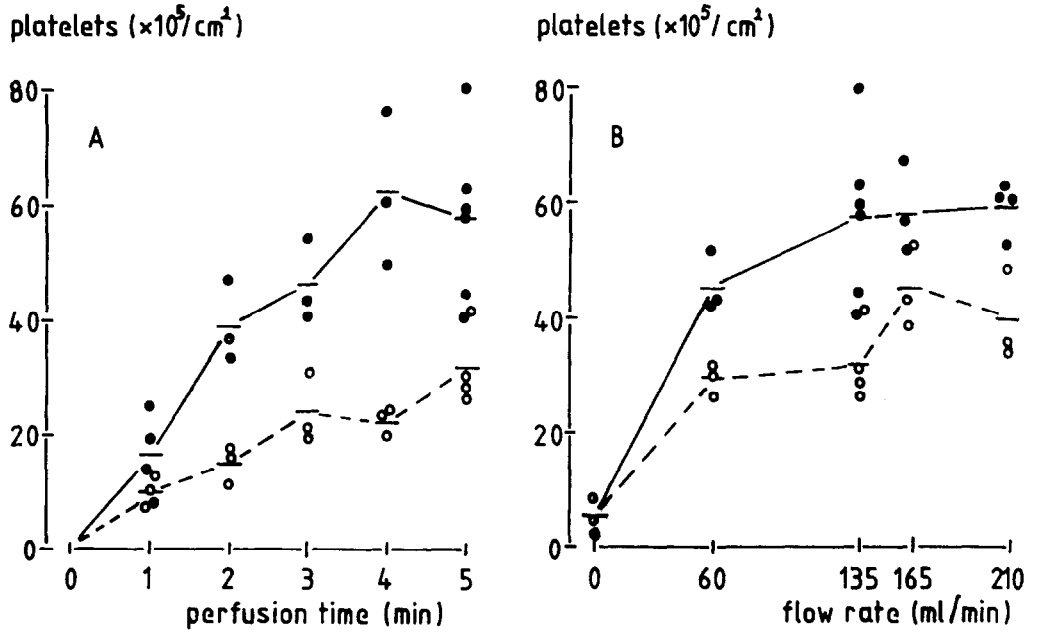

Fig. 5, A and B.

—— • steady flow, - - - o pulsatile flow.

A. Dependence of platelet adhesion on the perfusion time. The platelet count was 1.9 x 10^{11}/l plasma, the (average) flow rate 135 ml/min, and the red cell concentration 40%.

B. Dependence of platelet adhesion on the (average) flow rate. The perfusion time was 5 min, the red cell concentration 40%, and the platelet count 1.9 x 10^{11}/l plasma.

8. Platelet adhesion in normal and Von Willebrand plasma.
To compare the platelet adhesion kinetics in normal and Von Willebrand plasma in steady flow, the perfusion times were varied from 1 to 20 min, Fig. 6. After perfusion for 1 min no difference in platelet adherence in normal or Von Willebrand plasma was observed. Perfusion times ranging from 2 to 5 min resulted in on the average twice as many adherent platelets in normal plasma than in Von Willebrand plasma. However, by increasing the perfusion time, the difference in platelet adhesion in these two plasmas was minimized.

9. Morphometric evaluation of platelet-subendothelium interaction.
Pulsatile flow induced more contact platelets and less adherent platelets as compared to that observed in steady flow. No platelet aggregates were found.

The morphometric evaluation (Table III) was in accordance with the registrated ^{51}Cr-count, and showed that the registrated ^{51}Cr-count represent adherent platelets in both pulsatile and steady flow with 1.9 x 10^{11} platelets/l plasma. About half the number of contact platelets and approximately 2.5 times the number of adherent platelets found in pulsatile flow were observed in steady flow.

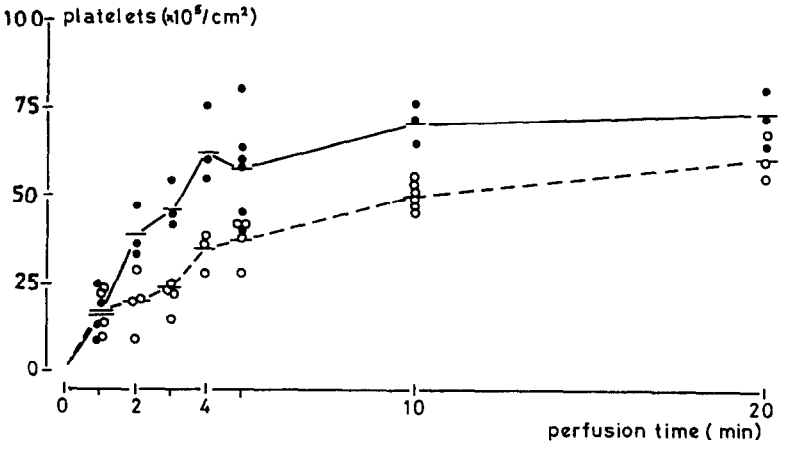

Fig. 6

Dependence of platelet adhesion in normal plasma and Von Willebrand plasma in steady flow on the perfusate time. ——— • normal plasma, - - - o Von Willebrand plasma. The flow rate was 135 ml/min, the red cell concentrations 40%, and the platelet count 1.9 x 10^{11}/l plasma.

10. Blood cells after perfusion.

The recovery of the platelets and the haemolysis were found to be similar in the two perfusion systems after perfusing 1.9 x 10^{11} platelets/l plasma and 40% red cells at 135 ml/min. Approximately 5% of the platelets were lost , and about 0.6% of the red cells were lysed after perfusion for 5 min Decreasing the perfusate volume resulted in increased haemolysis.

Table II

Platelet-subendothelial interactions.

Flow	% coverage			
	C	S	A	C/S
Pulsatility	6	19	25	0.32
-	4	17	21	0.24
-	5	18	23	0.28
Steady	2	55	57	0.04
-	2	57	59	0.04
-	2	63	65	0.03

The perfusate contained 1.9 x 10^{11} platelets/l plasma and 40% red cells. The perfusion time was 5 min and the (average) flow rate 135 ml/min.

DISCUSSION

Several approaches have been applied to investigate platelet interaction with artificial surfaces and arterial subendothelium in in vitro flow models (1, 21, 22). In the present investigation, the effect of the pulsatility of the flow on the platelet adhesion to human subendothelium was determined. The in vivo arterial circulation shows pulsatility in the aorta which is diminished throughout the arterial tree to nearly steady flow in the microcirculation (23 a). Backflow occurs only in the beginning of the ascending aorta, due to closure of the aortic valve and filling of the coronary arteries (23 b). The elasticity of the vessel wall modulates the flow amplitude and these properties may change by atherosclerosis or aging (23 c). Concerning the flow amplitudes in the two perfusion models, the one with steady flow may be comparable to the flow in the micro-circulation, where the shear rates are exceeding 800 sec^{-1}. The perfusion model with pulsatile flow is comparable to arterial flow, though the frequencies in our in vitro perfusion model are much higher than those in vivo. The greater number of adherent platelets at both edges of the vessel segment, in pulsatile and steady flow, may reflect differences in flow properties on the length of the vessel segment (Fig. 3). According to theoretical considerations, flow turbulence will occur at the edges of the artery (19), while at the middle part the flow is considered to be laminar (6). It was reported that the edge effects could be eliminated by modification of the rod (24), without affecting the adherence in the middle part. The influence of red cells (Fig. 4,B), flow velocity (Fig. 5,B), platelet count (Fig. 4,A), and perfusion time (Fig. 5,A) in both pulsatile and steady flow on the rate of platelet adhesion showed a similar tendency as in the Baumgartner perfusion model(9,14,25).The presence of red cell increases the radial platelet diffusivity in flowing blood (26), and thereby increases the rate of adhesion (14). The increased number of adherent platelets observed at increasing shear rate is presumed to be induced by an increase of radial platelet diffusivity (22, 27, 28). By increasing the platelet count, the observed increase of platelet adherence, also reported by others (29), reflects an enhanced rate of platelet-subendothelium collisions. Evidence for platelet aggregates were not found at the higher platelet counts (Table II) (7). However at subnormal platelet counts (1.9 to 2.5 x 10^{11}/l plasma) the steep increase of adherent platelets in pulsatile flow is apparently exerted by the influence of the platelets themselves on each other. A similar steep increase of adherence was also observed with platelets four times washed in the presence of EDTA (30) with a red cell concentration of 20% and platelet concentrations between 0.3 and 0.6 x 10^{11}/l plasma (8). The perfusion time in these experiments was 10 min.

It is evident that pulsatile flow at the frequencies applied (Table I) diminishes the rate of platelet adhesion as compared to that observed in steady flow. This difference can be explained partly from the relation between the the adhesion and the flow rate, which is a power function with a coefficient well below 1. From the data for steady flow given in Fig. 5, this coefficient is about 0.32, slightly lower than the value of 0.45 reported earlier by Turitto et al(29).In pulsatile flow,the increase in adhesion during the fast part of the pulse is relatively less than the decrease in the slow part. By graphical evaluation we found this effect to account for about half the difference between the adhesion in pulsatile and steady flow at 60 ml/min, but it is vanishingly small at 135 ml/min and higher flow rates.

Effects arising from the inclusion of a funnel in the system were excluded experimentally and therefore the difference has to be attributed to e.g. an impaired platelet attachment or spreading reaction due to shear stress variations in pulsatile flow. However, in a recent investigation (31), pulsatile flow was found not to reduce the platelet adherence to collagen

fibers, but the frequency applied was lower than that reported in the present study. Also, exposure of citrated rabbit blood to subendothelium of rabbit aorta in pulsatile and steady flow resulted in no significant difference in the platelet adherence (24). However the frequency and the average vessel wall shear rate were much lower than those applied by us.

Comparative studies of platelet-adhesion to human subendothelium in our perfusion models have to be carried out with a subnormal platelet count and short perfusion times. Otherwise, differences are rapidly obscured by saturation effects (in perfusion experiments with a physiological red cell concentration and vessel wall shear rates comparable to that found in the microcirculation).As an example, the difference in adhesion when platelets are perfused in normal and Von Willebrand plasma (Fig. 6) is most evident after 2 to 5 min perfusion. Increasing the perfusion time above 5 min minimizes the difference, in spite of a sub-normal platelet count.

The loss of platelets during the perfusions may be due to their adherence to the materials of the perfusion systems. No damaged or degranulated circulating platelets could be detected (results not shown). However the granules were localized in the center and the pseudopod-formation was evident indicating activation of the perfused platelets (32). The cause of haemolysis may be of mechanical nature, probably due to the rollers of the pump. The higher haemolysis found with the smaller perfusate volume may have been induced by the more frequent exposure of the red cells to the rollers.

In conclusion, the rate of platelet adherence in steady flow is larger than in pulsatile flow. The pulsatility and/or frequency may affect the platelet spreading on the vascular surface. The saturation effect has to be taken into account when comparative studies of platelet adherence are performed.

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