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Inhibition of PAF-induced aggregation of human PMNs and platelets by adenosine: in vitro investigations using a newly developed blood filtration system

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Abstract. An innovative filtration system permits the quantitative study of humoral and cellular interactions in flowing blood under the influence of mediators and inhibitors of inflammation. In an initial study it could be demonstrated in diluted human whole blood that PAF rapidly induced plugging of capillary-sized pores due to changes in shape and adhesivity of the PMNs and platelets. Adenosine proved to be a potent inhibitor. This nucleoside is increasingly produced in PAF-stimulated and filtrated blood, especially after its contact with cultured endothelial cells. In conclusion, these observations contribute to explain why, in the centre of a focus of inflammation in vivo, perfusion ceases while hyperaemia occurs in the adjacent marginal zone.

Abbreviations

PAF: platelet activating factor, FMLP: N-formyl-L-methionyl-L-leucyl-L-phenylalanine, PMN: polymorphonuclear leukocytes, EHNA: erythro-9-(2-hydroxy-3-nonyl) adenine, CAD: coronary artery disease.

Introduction

Although many new insights into the interactions between blood cells and the vascular wall in inflamed areas have been obtained through the systematic use of modern immunological and microscopical techniques, many unanswered questions remain, the resolution of which requires special in vitro methods that have yet to be developed. For example, it is not known to which extent the reorganisation of inherent leukocytic membrane structures or the formation of new ones induced by mediators of inflammation might modify the rheological behaviour of whole blood. A prompt enhancement of the adhesivity or a reduction in the deformability of the PMNs brought about in this way could, in particular in the early stage of inflammation, contribute greatly towards initiating the interaction of these cells with the cell adhesion molecules [1] of the stimulated endothelium. Also, not very much is known about the role played by the platelets or blood plasma in the early stages of the inflammatory process. Moreover, until today a phenomenon could not be explained that has been known to pathologists since the time of R. Virchow, namely, while in the centre of a focus of inflammation perfusion ceases, hyperaemia develops in the adjacent marginal zone.

Evidence is, however, accumulating to suggest that the complex cellular reactions and interactions in the vascular bed depend not only on numerous soluble activators, but also on inhibitors, and that it is the concerted action of all these various influences on all the blood cells and the vascular endothelium that decides whether the vessels in an organ will be perfused physio-

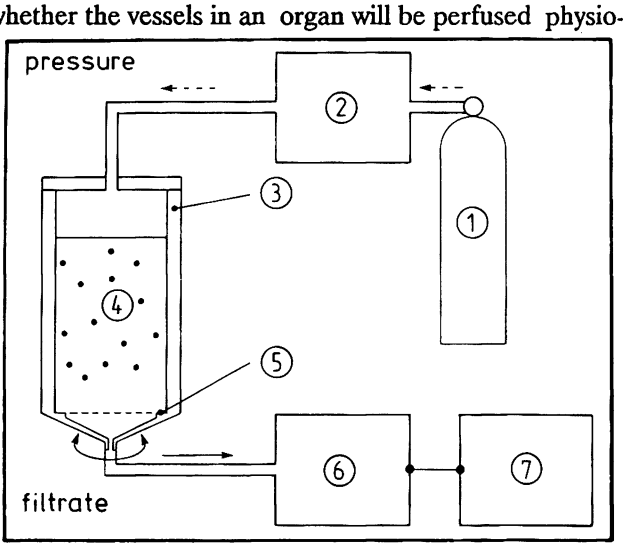

Fig. 1. Schematic diagram of the filtration system. 1: gas cylinder, 2: electronically regulated pressure device, 3: filtration chamber, 4: whole blood, 5: filter (thickness: 10 μm, pore diameter: 5 μm), 6: filtrate detector, 7: computer and plotter

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logically, or whether a thrombotic or inflammatory process will develop [2].

Unfortunately, accurate biochemical research into these interrelationships under in vivo conditions is limited by almost insurmountable methodological shortcomings. However, using a specially developed, versatile blood filtration system, we can now quantitatively measure the cell-specific effects and concentration-dependent actions of blood cell activators or inhibitors in flowing whole blood under largely defined in vitro conditions. This is described below for a) PAF, a typical inflammation mediator [3], and for b) adenosine which is liberated from the endothelial lining of blood vessels or produced there by extracellular degradation of adenine nucleotides [4].

Methods

Venous blood was freshly drawn from healthy donors, anticoagulated with citrate, and stored in stoppered and slowly rotated polyethylene tubes at 37°C until used. Prior to filtration, the blood samples were always diluted to a haematocrit of 6% with a physiologically balanced salt solution, and mixed with heparin (9 IU/ml).

Platelet-rich plasma was obtained by centrifugation of citrated blood (10 min at 1,500 rpm, Labofuge, Christ). PMNs were purified (95%) with the aid of defined Nycodenz solutions in accordance with the instructions of the manufacturer (Nycomed, Torshov, Norway).

The schematic diagram of the blood filtration system (recently developed in cooperation with the company of BOSCH-Wägetechnik GmbH, D-7455 Jungingen) is shown in Fig. 1. The blood cells were filtered through a polycarbonate filter (Nucleopore, Pleasanton, Calif.; thickness 10 µm, pore diameter 5 µm) in a pressure gradient (12 cm H_2O) kept constant by electronic means. The amount of filtrate was continuously determined gravimetrically with the aid of an electronic detector, and the results fed on-line to a computer and evaluated in accordance with the principle shown in Fig. 2.

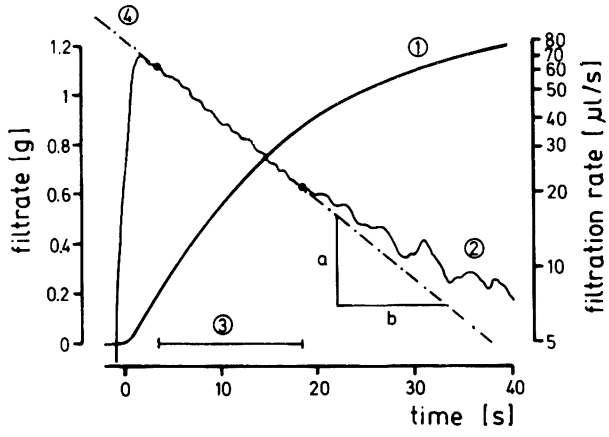

Fig. 2. Principle of the quantitative evaluation of the filtration data. Time courses of filtrate accumulation and filtration rate: curves 1 and 2, respectively; evaluation interval: 3; tangent: 4. A few seconds after the onset of filtration, the process is characterized by a phase in which a practically constant fraction of the filter pores is being plugged by PMNs and/or platelets per unit of time. The duration of this phase primarily depends upon the rapidity of activation of these cells, and the corresponding flow F through the filter can be described by the relationship $F = \alpha \cdot e^{-\beta t}$ (α: material constant of the filter; ß: plugging rate; t: filtration time). ß is determined by the slope of the tangent (a/b) drawn to curve 2. Occlusion rate $= (1-e^{-\beta}) \times 100$ [%/s]

After performance of the experiments, the filters were removed immediately, fixed and histochemically investigated. The influence of umbilical vein endothelial cells on the production of adenosine was investigated by passing PAF-stimulated blood samples through columns (Ø: 5 mm, length: 2 cm) packed with microbeads (Biosilone, Nunc, Roskilde, Denmark) on which confluent layers of this endothelial cell type had been established [5]. Adenosine was analysed according to HPLC-standard procedures [6].

Results

Under largely physiological conditions (constant pressure gradients, 37°C, 1.2 mM $CaCl_2$), heparinized whole blood readily passed through the capillary-sized pores of the measuring system at an almost constant, high flow rate (occlusion rate = 1.3 ± 0.32 %/s, n = 18). After preincubation with PAF, in contrast, the pores became progressively blocked. The calculated occlusion rate revealed a characteristic concentration dependence (Fig. 3). Analogous studies performed with purified suspensions of various blood cell types, the respective concentrations of which were adjusted to those found in correspondingly diluted whole blood, showed that the microrheological behaviour of the RBCs or lymphocytes was not influenced by PAF. In contrast, purified PMNs and platelets both occluded the filter pores in the presence of PAF, and the resulting dose-effect curves, taken together, could explain the curve obtained for whole blood in good approximation. Staining and microscopic examination of the filters finally proved

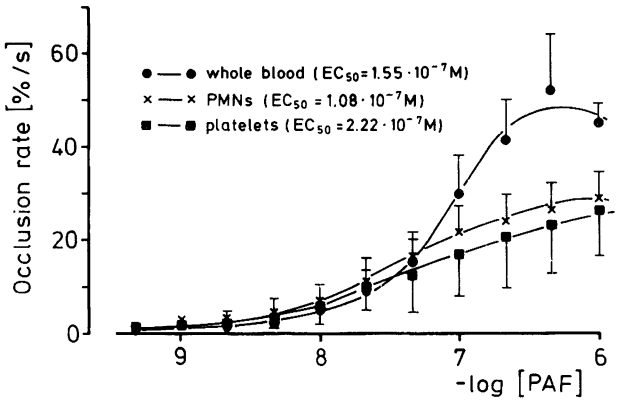

Fig. 3. Dose effect curves of PAF on the occlusion rate during filtration of whole blood, purified PMNs, or platelet rich plasma, respectively (details see text). n = 5, means ± SD. A significant increase in the occlusion rate during filtration of whole blood occurred already at a PAF-concentration of 2 nM ($p = 0.05$)

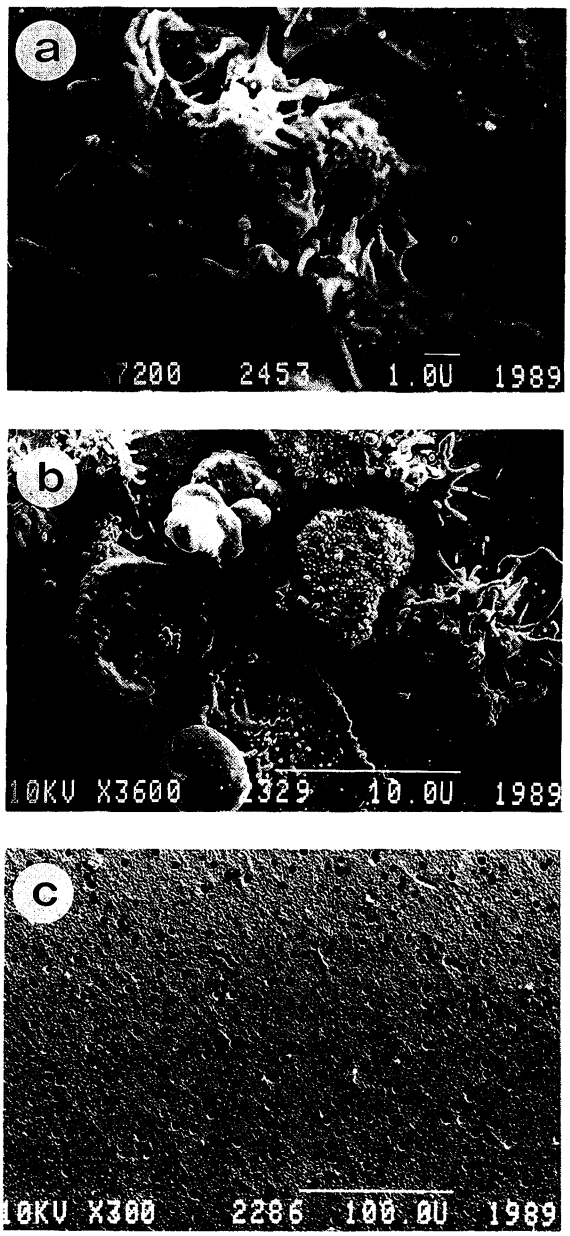

Fig. 4. Scanning electron micrographs of filters through which whole blood has been passed for 30 s in the presence of a) 2 nM, b) 200 nM PAF, and c) in the absence of PAF

that, in whole blood, PAF selectively stimulates only the PMNs and the platelets. During this process, these two cell species changed shape in a typical manner. On the basis of the EC_{50} values given in Fig. 3, it can be seen that PMNs are stimulated by appreciably lower concentrations of PAF than the platelets. Furthermore, under the influence of PAF, PMNs and platelets in whole blood interacted, and it were the resulting micro-coaggregates that finally plugged the capillary pores (Fig. 4a and b). The specificity of these interactions was underscored by the fact that in the absence of PAF, no cell aggregates were to be detected on the surface and in the pores of the biologically inert filters (Fig. 4c).

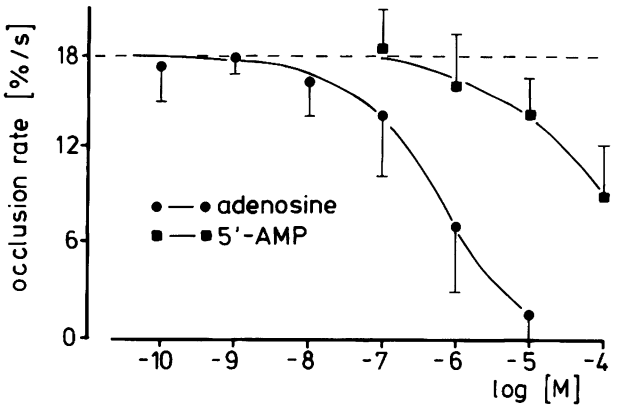

Fig. 5. Inhibition of occlusion rate induced by PAF (0.05 μM) by various concentrations of adenosine or 5'-AMP. Blood samples were preincubated for 90 s with the respective inhibitor, and for 60 s with PAF. n = 5, means ± SD. A highly significant decrease in the occlusion rate occurred already at 10^{-7} M adenosine and 10^{-6} M AMP, respectively ($p = 0.01$)

Adenosine appreciably suppressed the negative effect of PAF on the microrheological properties of whole blood, already at physiological plasma concentrations (approx. $2x10^{-7}$M), and even completely prevented it at higher concentrations (Fig. 5). 5'-AMP developed an appreciable inhibitory effect only at much higher concentrations. With the aid of sensitive HPLC techniques this "AMP-effect" could be traced back solely to adenosine, which was formed from 5'-AMP by the action of 5'-nucleotidase present on the cell membrane of lymphocytes and monocytes. Inosine proved to be completely inert.

As Fig. 6 reveals, the plasma adenosine concen-

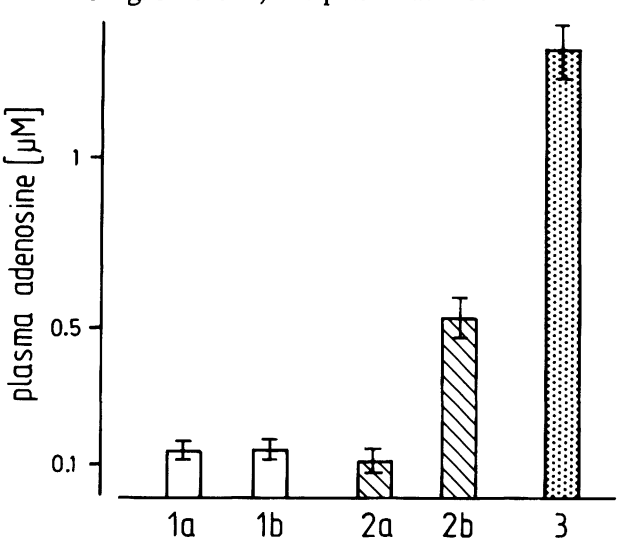

Fig. 6. Plasma adenosine concentration in (1) whole blood (mixed with 10 μM dipyridamol and 10 μM EHNA) in the absence of PAF a) prior, b) 1 min after filtration and immediate centrifugation (endothelial cells had no influence on these values); (2a,b) as in (1), but in the presence of PAF (1 μM); (3) as in (2b), but after subsequent passage during 1 min through a microcarrier column coated with endothelial cells. n = 4, means ± SD

tration of whole blood was unaffected by the filtration process, since the values prior and after passage of the pores were identical. However, blood samples which were stimulated with PAF contained appreciably higher adenosine concentrations after passage of the pores than before, due to stimulated release and degradation of adenine nucleotides from the platelets. Interestingly, if these samples were subsequently passaged through densely packed endothelial cell cultures, the adenosine concentration was further elevated. As relevant control experiments reveal, approximately 20% of this nucleoside fraction was released from the endothelial cells.

Discussion

Filtration techniques have been in use for some time to characterise the passive rheological behaviour of blood, mainly in the form of purified RBC suspensions [7]. However, for a reliable recording of active and rapid Ca^{2+}-dependent leukocyte and platelet reactions and interactions at a temperature of 37°C, the measuring devices and evaluation procedures described in the literature are not adequate. This technical limitation has now been overcome by the sensitive, electronically controlled filtration procedure we have developed. The haematocrit of 6% we selected is of the order of magnitude of microhaematocrit values measured in vivo [8].

Scanning electron microscopic examinations revealed that PAF can stimulate PMNs and platelets to effect highly complex changes in their shape, size, rigidity, adhesivity, and aggregability. As we have demonstrated, these effects occurred statistically significant even at low PAF concentrations around 2 nM. Such values can be found in arterial blood of CAD-patients [9], and are probably even higher in blood under conditions of septicaemia.

In other studies [10], we have been able to show that FMLP, another mediator of acute inflammation, selectively activated the PMNs in whole blood, while 5'-ADP stimulated only the platelets. However, in these cases, too - probably mediated by products (e.g. oxygen radicals) released secondarily by the stimulated cells - activation of the respective other cell species soon occurred. This close cooperation between PMNs and platelets has to date received but little attention in studies of inflammatory processes.

In vivo the concentration of PAF is greatest at the centre of an inflammatory focus due to its release from activated leukocytes and endothelial cells [3]. The moment PAF accumulates in the microcirculation of the inflamed area, it should rapidly induce similar cellular reactions as we could demonstrate in vitro. The resulting plugging of the capillaries by the blood cells and the decrease of flow are then probably very important preconditions for the PMNs and monocytes to attach to the vascular wall and to bind to the initially only weakly expressed adhesion molecules at the endothelium [1].

The PAF-activated platelets, on the other hand, release ADP and ATP, which can be dephosphorylated to adenosine in the intravascular space, but rapidly only by the luminal ecto-nucleotidases of the vascular endothelium. As the blood is distributed within more and more microvessels downstream of the focus of inflammation, it is simultaneously being brought into contact with an ever increasing endothelial surface. In this way, it would be possible in vivo for considerable concentrations of adenosine to build up in the very surrounding of the primary focus. Together with other typical endothelial cell release products, such as prostanoids (PGI_2, PGE_1, PGE_2) and nitric oxide (NO) this could prevent PMNs and platelets from entering the remaining circulation in an activated state. All these compounds are potent vasodilators, and might thus also be responsible for the hyperaemia that is typically observed in the marginal zone surrounding the focus of an inflammation.

Finally, mediators of acute inflammation, like PAF [3], and endothelium-derived inhibitors of leukocytes and platelets, like adenosine [6], have only a short half-life in circulating blood. This could contribute to explain why, immediately outside the zone of inflammation, physiological perfusion and regulation of the blood vessels are again observed.

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