Involvement of ecto-ATPase and extracellular ATP in polymorphonuclear granulocyte-endothelial interactions

Galina F. Sud'ina[a,*], Olga K. Mirzoeva[a], Svetlana I. Galkina[a], Marina A. Pushkareva[a], Volker Ullrich[b]

[a] *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia*
[b] *Faculty of Biology, University of Konstanz, 78457 Konstanz, Germany*

Received 12 January 1998

Abstract The adhesion of human polymorphonuclear granulocytes (PMN) with confluent human endothelial cells (line EAhy926) and with solid substrate coated by collagen and fibronectin (Fn) was studied by phase contrast microscopy and by the measurement of myeloperoxidase activity. The ecto-ATPase inhibitors suramin and Reactive Blue 2 (RB2) more than doubled the adhesion of PMN to endothelial cells. The cells hydrolyzed added ATP and this reaction was inhibited by suramin and RB2. The degree of ATP hydrolysis during PMN adherence depended on solid substrata and decreased in the order: non-stimulated endothelial cells, TNF-stimulated endothelial cells, collagen-coated surface, Fn-coated surface. In the same order adherence increased. The endogenous level of extracellular ATP in the PMN-endothelial coculture was around 25 nM.

We conclude that PMN-endothelial adhesion is counteracted by an ecto-ATPase or by ATP receptors with ATPase activity. Such interactions may play a role in PMN rolling and diapedesis as well as in the pathophysiology of PMN activation by an anergic endothelium.

Key words: Adhesion; Ecto-ATPase; ATP; Collagen; Fibronectin; Suramin

1. Introduction

Leukocyte recruitment to the tissue sites of inflammation requires a complex interaction between circulating polymorphonuclear leukocytes (PMN) and the endothelium including the subendothelial matrix [1]. What is considered as a physiological defence mechanism against invading microorganisms can under severe ischemia-reperfusion or shock conditions turn into a self-destructive mechanism (of an organ) and thus may be lethal to an organism [2–4]. Therefore the mechanisms of PMN adhesion to the endothelium and the subsequent steps in diapedesis have been extensively studied in order to find pharmacological targets.

Both leukocytes and the endothelium play an active part in these processes which are governed by adhesion molecules and their receptors [5–8]. Integrins, selectins and proteins of the immunoglobulin gene superfamily mediate the various stages of adhesion which turns out to be an active and energy-utilizing process. Since ATP is required to synthesize, express and degrade components of the adhesion mechanism it explains why cooling of tissues prevents destructive processes in traumatic or ischemia-reperfusion events [9–12]. On the other hand under physiological conditions energy is also required to prevent leukocyte adhesion to the endothelium through synthesis of nitric oxide or prostacyclin [13,14], which by intracellular cGMP- or cAMP-dependent phosphorylations actively counteract a sticking of PMN to the endothelium. It had even been suggested that ATP directly controls adhesion by ATP receptors or ecto-ATPases [15].

In this paper we made use of ecto-ATPase inhibitors and ATP receptor antagonists to look for a correlation between extracellular ATP hydrolysis and neutrophil adhesion during neutrophil interaction with different surfaces. We found microscopic and biochemical evidence that indeed the ecto-ATPase and/or ATP receptor antagonists suramin and Reactive Blue 2 (RB2) increase adhesion of human PMN to confluent endothelial EAhy926 cells. The increase of adhesion to suramin treatment correlated with a decrease of extracellular ATP hydrolysis to this treatment. The fact that suramin and RB2 acted differently on the adhesion of PMN to fibronectin allowed to conclude on a rather specific action of ATP.

2. Materials and methods

The endothelial permanent cell line EAhy926 was a kind gift of Dr. Cora-Jean S. Edgell (University of North Carolina, USA). Dulbecco's modified Eagle's medium (DMEM), HAT-supplement, L-glutamine, penicillin, streptomycin were purchased from Gibco (Eggenstein, Germany), fetal calf serum (FCS) and collagen type I from Boehringer Mannheim (Mannheim, Germany), human plasma fibronectin (Fn) from Calbiochem (San Diego, CA, USA). Tumor necrosis factor was from Falcon (Franklin Lakes, NJ, USA). Ficoll-Paque was purchased from Pharmacia (Uppsala, Sweden). HEPES, ortho-phenylenediamine were purchased from Fluka (Deisenhofen, Germany). ATP, ADP, AMP, adenosine, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), adenosine deaminase, ouabain, trypsin-EDTA were purchased from Sigma (Deisenhofen, Germany).

2.1. Preparation of biological surfaces for leukocyte adhesion

Tissue culture plastic 24-well plates were coated with collagen (75 μg/ml, for 24 h) or Fn (3 μg/ml, for 3–5 h). Collagen-coated culture plates were washed with DMEM with 0.5% FCS and used for adherence and for endothelial cell culture. Before use dishes were thoroughly washed with phosphate-buffered saline (PBS).

2.2. Cell culture conditions and treatment

Endothelial cell permanent culture EAhy926, passages 179–185, maintained in HAT-supplemented (100 μM hypoxanthin, 0.4 μM aminopterin, 16 μM thymidin) Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, 4 mM L-glutamine, 10 U/ml penicillin,

*Corresponding author. Fax: +7 (95) 939 3181.
E-mail: sudina@bk408.genebee.msu.su

Abbreviations: PMN, polymorphonuclear granulocytes; TNF, tumor necrosis factor; RB2, Reactive Blue 2; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; Fn, fibronectin; HBSS, Hanks' balanced salts solution with 10 mM HEPES, pH 7.4

PII S0014-5793(98)00102-1

10 μg/ml streptomycin, and 10% FCS, as recommended by Edgell et al. [16]. Cultures were passaged weekly using trypsin-EDTA solution (500 BAEE units trypsin and 180 μg EDTA/ml in PBS), and seeded on 24-well plates, or 75 cm^2 culture flacons, coated by collagen. One or 2 days before the experiments, the medium was exchanged for DMEM with 0.5% FCS. When indicated, the cells were activated by TNF-α, added to this medium for 6 h at a concentration 8 ng/ml.

2.3. PMN isolation procedure

PMNs were isolated from freshly drawn donor blood on a bilayer gradient of Ficoll-Paque (1.077 and 1.125 densities). Granulocytes were washed twice in PBS and resuspended in Hanks' balanced salts solution (HBSS) with 10 mM HEPES, pH 7.4.

2.4. Phase contrast microscopy of cell cultures

PMNs were added to control or activated (TNF-α, 8 ng/ml, 6 h) EAhy926 cells grown to monolayer on collagen-coated cover slips, for 20 min at 37°C. After washing with PBS, the samples were examined with an Opton-III Microscope (Zeiss, Germany).

2.5. PMN adhesion assay

Myeloperoxidase activity was used as a marker enzyme to measure polymorphonuclear leukocyte (PMN) adherence to endothelial cells and to collagen- or fibronectin-coated surfaces. PMNs were added to 24-well culture plates containing an endothelial cell (EAhy926) monolayer, or coated by fibronectin or collagen, (5×10^5 PMNs per well, in 500 μl of HBSS medium with 10 mM HEPES, pH 7.4). After 30 min incubation in a CO_2 incubator at 37°C to allow leukocyte adherence, wells were washed 2 times with 500 μl PBS solution, for removal of non-adherent PMN. The amount of adherent PMN was measured by the addition of detergent and a peroxidase substrate, as described [17,18]. 300 μl of a solution of 5.5 mM ortho-phenylenediamine and 4 mM H_2O_2 in buffer (67 mM Na_2HPO_4, 35 mM citric acid, 0.1% triton X-100, pH 5), were added to each well, and after 4 min, the reaction was stopped by the addition of the equal volume of 1 M H_2SO_4. Standard dilutions of PMN with or without tested compounds were used for calibration.

2.6. Ecto-ATPase activity assay

Hydrolysis of exogenously added ATP by the endothelial cell culture, by the PMN-endothelial cell coculture, and by PMNs on collagen or fibronectin, was determined by HPLC analysis of the supernatants collected at timed intervals and assayed for nucleotide concentrations. Cell incubations were performed in HBSS/HEPES medium. Suramin (100 μM), when indicated, was added to the medium before the PMN. PMNs were added to endothelial monolayers, or to collagen- or Fn-coated surfaces in 24-well plates, 5×10^5 cells/well. ATP at concentrations of 100–1000 μM was added after PMN, and plates were incubated at 37°C in a CO_2 incubator. At regular intervals, 250 μl of supernatant was taken and mixed with 50 μl of a solution consisting of 50 mM EDTA and 0.9% NaCl to stop the reaction. The amount of nucleotide in the dish without cells was also measured as a control. Adenine nucleotides and adenosine in samples were determined by reversed phase HPLC on C18 (25 cm ×4.6 mm) 5 μm column. After 7 min elution with buffer A (2 ml 85% H_3PO_4 in 1000 ml H_2O, titrated to pH 5 with NH_4OH), fraction B (H_2O/acetonitrile, 1:1) was increased to 50% during 10 min. Nucleotides were detected at 254 nm.

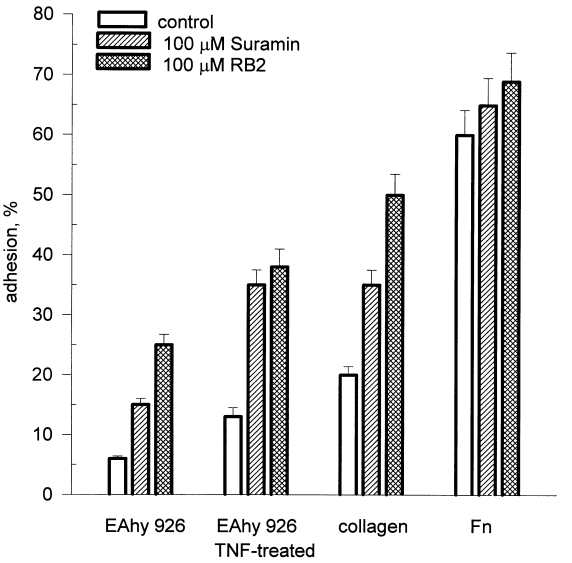

Fig. 1. Dependence of PMN adherence to endothelial EAhy926 cells, and to collagen- and fibronectin-coated surfaces on Suramin and Reactive Blue 2. Suspension of PMNs 10^6/ml incubated on various biological surfaces with or without Suramin and RB2 in incubation medium. After 30 min at 37°C, non-adherent leukocytes were washed out by rinsing.

2.7. Release of ATP, NO and prostacyclin

PMNs were added to 24-well culture plates, with endothelial cell (EAhy926) monolayers, or coated by fibronectin or collagen, 5×10^5 PMNs per well, in 500 μl of HBSS medium with 10 mM HEPES, pH 7.4. After 30 min incubation in a CO_2 incubator at 37°C for leukocyte adherence, plates were centrifuged at $2000\times g$ for 3 min. 200 μl aliquots of supernatant were mixed thoroughly with an equal volume of icecold 4 mM EDTA and 20 μl of 0.1 M KOH adjusted pH 7.7. The samples were centrifuged at $2000\times g$ for 3 min and the supernatant was stored at −70°C until analysis. The concentration of ATP in the samples was determined by an ATP bioluminescent assay kit (Sigma) according to the instructions of the manufacturer. Suramin interfered in this assay. NO was measured by the Griess test [19] and prostacyclin formation with a 6-keto-prostaglandin $F_{1\alpha}$ ELISA test [20].

3. Statistical analysis

Results are given as means ± S.E.

4. Results

4.1. ATPase inhibitors and PMN adhesion

Table 1 shows the effect on adhesion of PMN when the coculture was supplemented with the mitochondrial uncoupler

Table 1
Effects of different ATPase inhibitors and ATP on adhesion of PMNs to endothelial cells and to collagen

	Adhesion, %	
	PMN adhesion to EAhy926 cells	PMN adhesion to collagen
Control	6.8 ± 1.2	20 ± 2.0
Oligomycin, 10 μM	7.2 ± 1.0	22 ± 2.3
Ortho-vanadate, 200 μM	7.0 ± 1.1	20 ± 1.8
Ouabain, 200 μM	7.0 ± 1.0	26 ± 3.0
Suramin, 100 μM	15 ± 3	35 ± 5
Reactive Blue 2, 100 μM	25 ± 2	50 ± 5
ATP, 10 μM	9.1 ± 1.5	23 ± 2

A suspension of PMNs (10^6/ml) was added to a non-stimulated (control) endothelial monolayer, or to collagen-coated surface. Indicated compounds were added to HBSS incubation medium before PMNs. After 30 min incubation at 37°C, non-adherent leukocytes were washed out by rinsing.

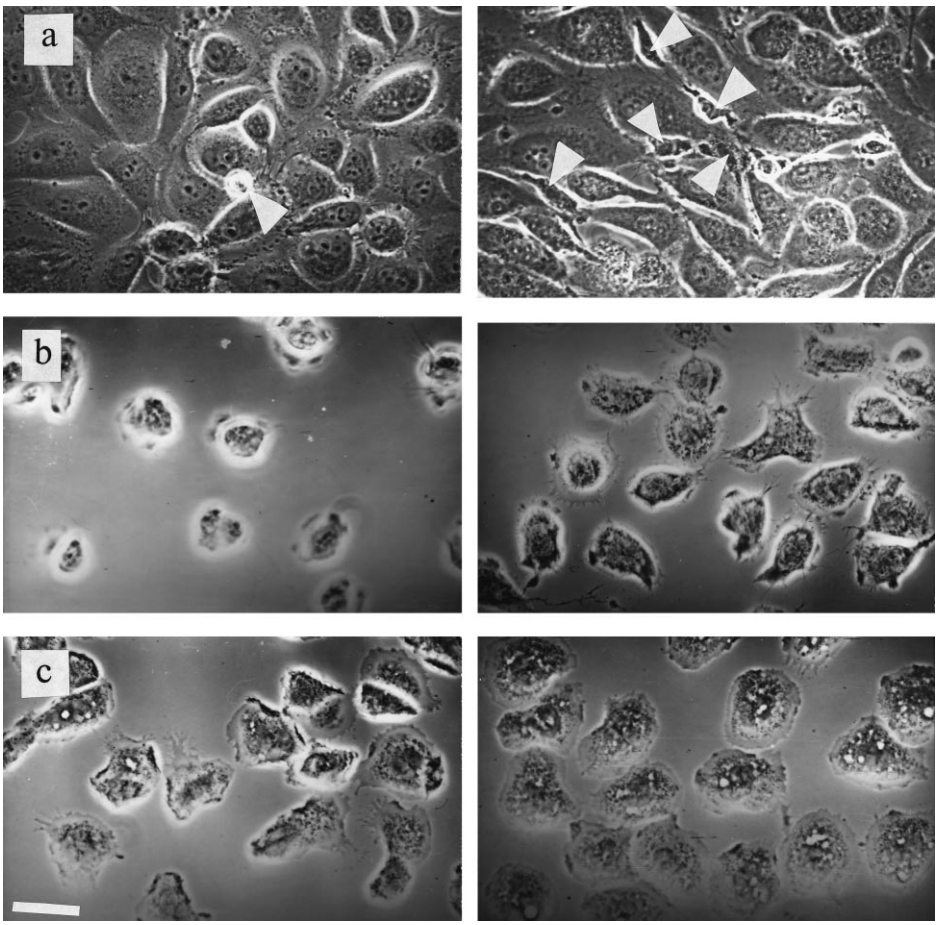

Fig. 2. a: Microscopy of PMN-endothelial cell coculture (arrows indicate PMNs) in the absence (left) and in the presence of 100 μM suramin. b and c: Microscopy of PMNs on collagen- and on fibronectin-coated surfaces, in the absence (left) and in the presence of 100 μM suramin. Bar, 10 μm.

oligomycin, the inhibitor of ion pumping ATPases, Na-orthovanadate and the Na/K-ATPase blocker ouabain. No significant effects were seen. But suramin and RB2, which have been described as inhibitors of ecto-ATPases and/or P2-purinoceptor antagonists caused a strong adhesion of PMN to endothelial cells and to collagen (Figs. 1 and 2). Both agents did not affect the adhesion to fibronectin (Figs. 1 and 2). Exogenously added ATP increased adhesion of PMN to endothelial monolayers and to collagen (Table 1). Phase contrast microscopy of PMN-endothelial cocultures in the presence of suramin showed flattened and spreaded PMN adhered to the intercellular space (Fig. 2).

4.2. Extracellular ATP hydrolysis during PMN adhesion

When ATP hydrolysis was measured in cocultures of PMN and EAhy926 cells differences were found which correlated inversely with the adhesion process. An HPLC profile of the ATP hydrolysis products with and without suramin is shown in Fig. 3. Because of the presence of ADP hydrolysis and AMP-5′-nucleosidase activity, the ratio of ADP+AMP+adenosine to ATP has been used as a measure of ATPase activity. Controls had a high activity of about 20% which decreased dramatically to 5–6% in the presence of suramin and RB2 (for RB2 data not shown).

One possible explanation for these results could have been the production of adenosine from ATP and the subsequent activation of adenosine receptors. After a 30 min incubation of 100 μM ATP with a PMN-human umbilical vine endothelial cell coculture about 4–5 μM adenosine could be measured, the level of extracellular adenosine was very low in PMN-EAhy926 cell coincubations, and Fig. 4 shows that adenosine could only partly reverse the suramin and RB2 effect on adhesion. The presence of adenosine deaminase (up to 1 U/ml) was without effect but EHNA, an inhibitor of adenosine deaminase (Fig. 3A), raised the adenosine concentrations to a detectable level and also decreased the RB2 induced adhesion process (Fig. 4).

If extracellular ATP directly would be responsible for a decreased adhesion in the coculture system distinct levels of ATP should be detectable under steady state conditions. We found that controls built up an ATP level of 13 nM which further increased to 25 nM in the presence of RB2. On a collagen surface a concentration of 10 nM and on a fibronectin surface a concentration of 6 nM were found, obviously originating from a secretion by PMN.

An alternative explanation of the data would be possible by an increase of NO or prostacyclin production by the EAhy926 cells. However, no stimulation of NO synthesis and no enhancement of 6-keto-$PGF_{1\alpha}$ formation could be detected.

5. Discussion

Under normal conditions of blood flow and average oxygen tension the vascular system uses several mechanisms to avoid

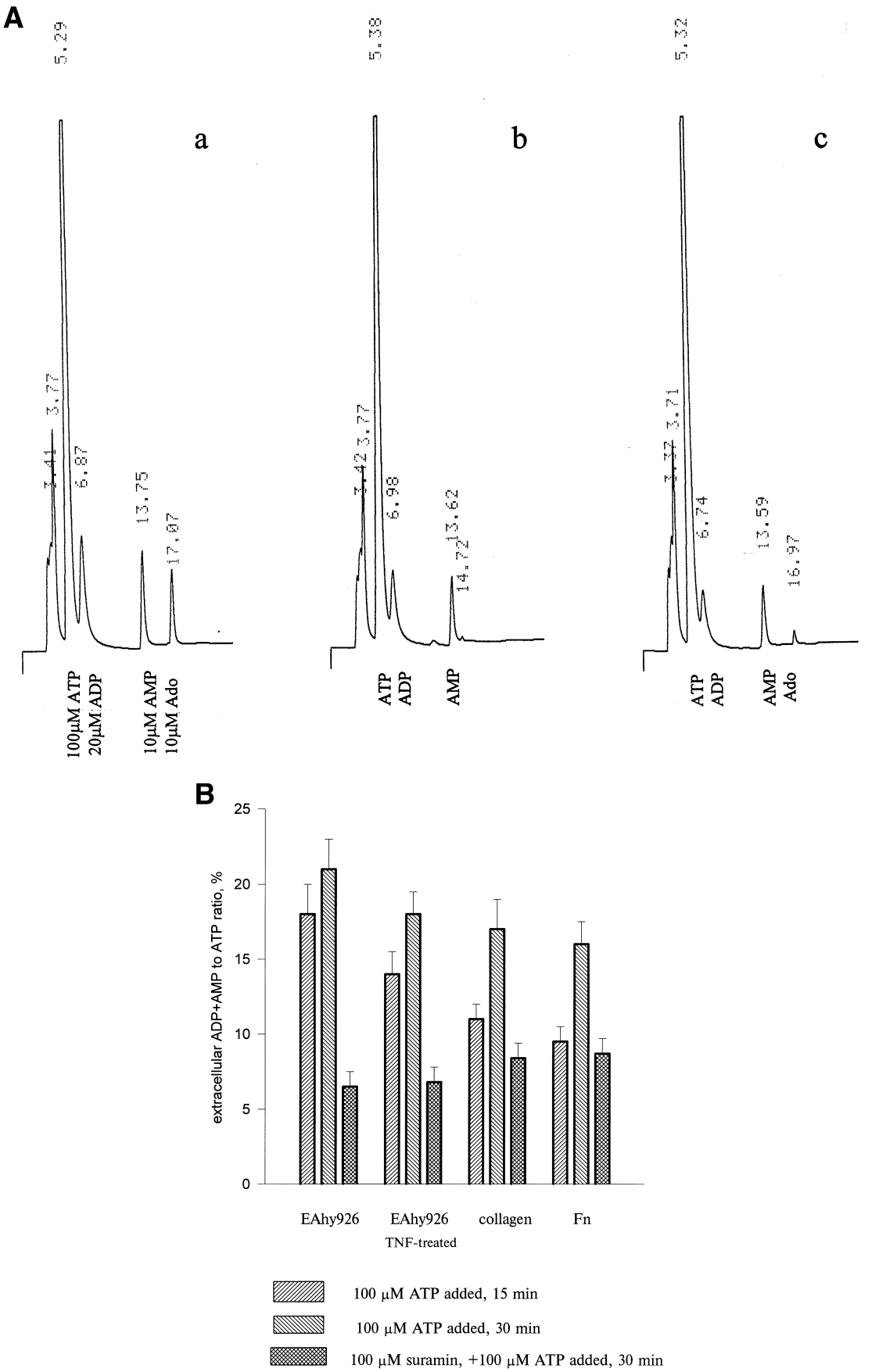
A
a
b
c
5.29
3.41
3.77
6.87
13.75
17.07
5.38
3.42
3.77
6.98
14.72
13.62
5.32
3.32
3.71
6.74
13.59
16.97
100μM ATP
20μM ADP
10μM AMP
10μM Ado
ATP
ADP
AMP
ATP
ADP
AMP
Ado
B
extracellular ADP+AMP to ATP ratio, %
0
5
10
15
20
25
EAhy926
EAhy926
TNF-treated
collagen
Fn
100 μM ATP added, 15 min
100 μM ATP added, 30 min
100 μM suramin, +100 μM ATP added, 30 min

Fig. 3. A: Extracellular ATP hydrolysis during PMN adhesion to endothelial cell EAhy926 monolayer. HPLC profiles of the products of ATP hydrolysis: a: the standard mixture of ATP, ADP, AMP and adenosine (Ado); b and c: the supernatants from PMN-endothelial cell coincubations 30 min at 37°C with 200 μM ATP, with (c) or without (b) 2 μM EHNA. B: Extracellular ATP hydrolysis during PMN adhesion to different surfaces, effect of suramin. Extracellular nucleotides in PMN incubations on different surfaces with 100 μM exogenously added ATP determined by HPLC. Data are presented as the ratio of products of ATP hydrolysis (ADP and AMP were determined, Ado was below detection level in these incubations) to ATP concentration in the supernatants after 15 and 30 min at 37°C, with or without suramin in HBSS incubation medium.
←

sticking of blood cells to the vessel wall. The endothelium plays an active part in this process by secreting prostacyclin, nitric oxide (NO) or the still uncharacterized endothelium-derived hypopolarizing factor (EDHF) in response to shear stress or humoral factors. It also has been suggested that ATP secretion [21] from endothelial cells acts in the same direction through ATP receptors which again cause the release of NO and prostacyclin [13,22,23] and thus prevent PMN adhesion. However, no increases of NO production or prostacyclin synthesis in EAhy926 cells were detected in the presence of ATP.

Our present results allow an additional explanation by assuming the involvement of ecto-ATPases. Based on previous reports on the inhibition of such ATPases by suramin and RB2 [24–26] we could show that blocking of ecto-ATPases leads to an increased adherence of PMN to a confluent endothelial cell culture. From this we conclude that an active ATPase on endothelial cells keeps PMN in a state of loose attachment whereas a decrease of ATPase activity in the presence of suramin or RB2 causes a transition to a tightly attached state. This is further supported by measurements of ATP breakdown, which was inversely correlated with the adhesion process. Suramin and RB2 block this ATPase activity. Collagen-coated surfaces but not those coated with fibronectin behaved similarly to endothelial monolayers suggesting that collagen receptors on PMN may also participate in the anti-adhesive process.

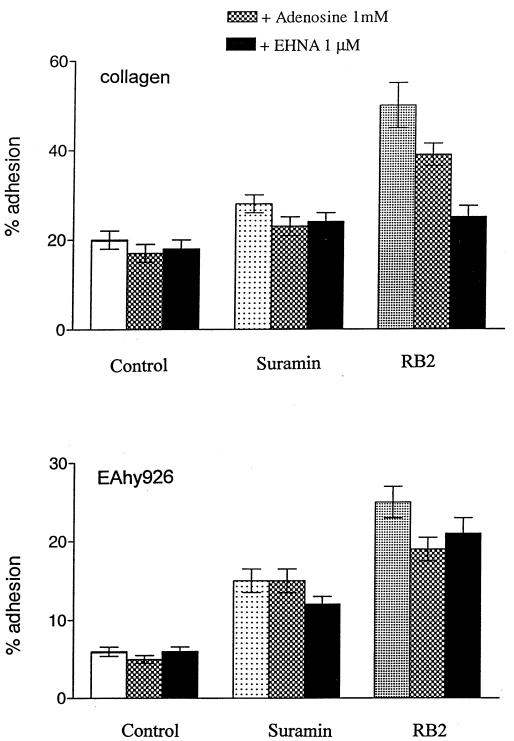

Fig. 4. Effect of adenosine and EHNA on suramin- and RB2-stimulated PMN adhesion to collagen and endothelium. Suramin, 100 μM, RB2, 100 μM, adenosine, 1 mM, or EHNA, 1 μM added to HBSS incubation medium before the addition of the suspension of PMNs 10^6/ml. After 30 min at 37°C, non-adherent leukocytes were washed out by rinsing.

It is known that vascular endothelial cells [27] and PMNs [25] are equipped with ecto-ATPases and 5′-nucleotidases, the key enzymes for the metabolism of adenine nucleotides. The detailed mechanisms by which ATPases counteract the adhesive process are not known. The hypothesis that the cell adhesion molecule CAM 105 is itself an ecto-ATPase [28] found no support by an investigation carried out by Stout and Kirley [29]. It is intriguing, however, to assume that ATP extruded from the cells exerts a signalling function between PMN and the endothelium and that ceasing of the outward transport leads to tight adherence of PMN in the intercellular space as observed in the present experiments.

Whether the transducing system is mainly a receptor or an ATPase that uses the chemical energy for active anti-adhesive processes is still not clear. It even becomes more complex when the further steps in ATP degradation are also considered. Except an ecto-ATPase there exists an ecto-5-nucleotidase generating adenosine which affects cell functions through purinoceptors. These receptors are subdivided into two classes: P1 with an agonist potency order of adenosine > AMP > ADP > ATP and P2 with a reverse ranking [30]. P1 receptors are divided into A1 and A2 subtypes [31], and adenosine, possessing high affinity to adenosine A2 receptors is known to inhibit neutrophil adhesive interactions [32]. In the light of this interaction between ATP and the purinergic receptors the hypothesis of Cunningham et al. [33] that ecto-ATPase may serve as a switch controlling extracellular ATP concentration and ligand accessibility to P1- and P2-purinoceptors, also seems to be attractive. As the main physiological role for endothelial ecto-nucleotidases their antithrombotic activity has been postulated [34] by their ability to hydrolyse the pro-aggregatory ADP [35]. That the formation of adenosine as the end product of ATP hydrolysis plays an important role in decreasing neutrophil adhesion, is supported by many investigations [32,36,37]. Also according to our data, adenosine could participate in preventing adhesion, but adenosine and the adenosine deaminase inhibitor EHNA only partly reversed the effect of RB2 on neutrophil adhesion to endothelial cells and to collagen, though EHNA increased the adenosine concentration in incubations up to micromolar levels. Adenosine can inhibit adhesion of neutrophils in concentrations as low as 10 nM [38]. Since this is well below the HPLC detection level, we cannot exclude that low amounts of adenosine were formed and supported the low basal adhesion in our PMN-EAhy926 cocultures.

Irrespective of the chain of events starting from ATP secretion it seems established that extracellular ATP plays an active role in the anti-adhesive mechanism between the endothelium and PMN, and probably also other blood cells. To support

this hypothesis it was important to detect extracellular ATP in our system. Indeed the medium contained 25 nM ATP in the coculture and 10 nM after a 30 min incubation of PMN with collagen surfaces. These levels amount to those found after the same time interval in a HL60 promyelocyte cell culture [39].

Taken together we suggest from our data that extracellular ATP can effectively counteract adhesion by a process that is sensitive to suramin and RB2, which have been reported as inhibitors of ecto-ATPase activity. Further studies must clarify the complex interplay of the presumed ecto-ATPase with ATP and adenosine receptors.

Acknowledgements: This work was supported by the Volkswagen Stiftung (Germany) and the Russian Foundation for Basic Research.

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