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Effect of adenosine derivatives on *in vitro* thrombus formation induced by shear stress

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ABSTRACT

Background and Objective. **Shear-stress is considered to be the first event of platelet aggregation *in vivo* and platelet adhesion may be enhanced under pathologic conditions (e.g. arterial occlusion).**

Design and Methods. **We wanted to test the effect of adenosine derivatives on platelet aggregation induced by shear-stress. By increasing platelet cAMP adenosine derivatives inhibit platelet activation. This in turn leads to P-selectin (CD62P) exposure, which is known to play a fundamental role in the binding of platelets to leukocytes. This gives rise to thrombus formation.**

Results. **The levels of cAMP (pmol / mL) prior to and after treating blood with the following compounds were respectively: PGE1 4.67±0.29, 9.33±0.58; SP64 5.2±0.34, 6.83±0.52; 2-Cl-adenosine 5.83± 0.58, 7.45±0.55; NECA 7.00±2.29, 8.00±1.76.**

Interpretation and Conclusions. **High shear rate was studied using a filteraggregometer which could be a good test for analyzing what happens under physiologic conditions compared to other systems in which platelet aggregation only occurs after adding aggregating agents (Born aggregometer).**

Key words: vWF, von Willebrand's factor; cAMP, cyclic adenosine monophosphate, adenosine derivatives, P-selectin, filteraggregometer

Platelet adhesion and aggregation play crucial roles in hemostasis. Platelets contribute to maintaining the normal circulation of blood through the preservation of vascular integrity and the control of hemorrhage after injury.[1,2] Shear-stress is considered to be the first event in the aggregation process *in vivo*.[3] The highest shear rate occurs in small arterioles having a 10-50 µm diameter. The response of adhesion mechanisms to high flow conditions may be even more important in pathologic conditions associated with the occurrence of acute arterial occlusion.[4,5] High shear-stress platelet aggregation depends on von Willebrand's factor (vWF), platelet glycoprotein complexes GP Ib/IX/V, integrin $\alpha_{IIb}\beta_3$,[6] and intracellular Ca^{++}.[7-10]

Platelet-leukocyte adhesion represents an important step in hemostasis and thrombosis.[11-14] It was shown that the interaction between platelets and polymorphonuclear leukocytes is through exposed platelet P-selectin (CD62P), and this represents the first step in platelet-leukocyte thrombus formation. This phenomenon was prevented by an anti-P-selectin antibody indicating that the interaction between platelets and leukocytes is P-selectin dependent. Moreover, it has been demonstrated that increasing cyclic adenosine monophosphate (cAMP) in platelets can block the possible exposure of P-selectin.[14,15] Interestingly, patients with acute myocardial infarction have increased leukocyte-platelet adhesion,[16] and in patients undergoing PTCA for myocardial infarction an increase in platelet activity has been observed.[17]As a consequence in these situations the binding of leukocytes to platelets is increased.

Among the compounds with antiplatelet activity that have been tested by shear-induced platelet aggregation, ASA proved to be ineffective,[18,19] while ticlopidine and PGE1 were seen to inhibit platelet aggregation.[20] Several other platelet antiaggregating drugs, including adenosine, have also been identified.[21] We therefore wanted to test the effects of shear on platelet function using the system described by O'Brien and Salmon.[22] These authors demonstrated that leukocytes also took part in thrombus formation in the filter. Tests were carried out in the presence or absence of PGE1 and several potent adenosine derivatives, thought to inhibit platelet aggregation by increasing cAMP just like adenosine, but at submicromolar concentrations.[23] One of these is 2-Cl-adenosine, but we also used adenosine-5'-N-ethyluronamide (NECA), the prototypical A_2 agonist and

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two NECA derivatives, SP50 and SP64, having a hydroxy and a cyano group on the side chain, respectively. These two latter compounds were synthesized because of the therapeutic potential of NECA in the treatment of cardiovascular diseases. They were also studied in binding and functional assays to test their affinity for A_2 adenosine receptors,[24] which are found mainly in blood vessels and platelets. NECA is not very A_2 selective.[25]

Design and Methods

Samples

Blood samples (20 mL) were collected in hirudin 500 U/mL from 25 healthy volunteers who had not made use of antiplatelet drugs for the previous 15 days. Untreated blood from the same subjects was used as control.

Compounds tested

The following inhibitors of platelet function were used. The final concentration is indicated in brackets.

- PGE1 (10 µM);
- 2-Cl-adenosine (20 µM);
- (5-N-ethylcarboxamido-adenosine) or NECA (1 µM);
- {N-Ethyl-1'deoxy-1'-[6-amino-2-(5-cyano-1-pentyn-1-yl)-9H-purin-9-yl]-β-D-ribofuronamide} or SP64 (0.5 µM);
- {N-Ethyl-1'-deoxy-1'-[6-amino-2-(3-hydroxy-1-pentyn-1-yl)-9H-purin-9-yl]-β-D-ribofuronamide} or SP50 (0.1 µM).

All the samples were tested after 5 minutes of incubation at room temperature.

As far as NECA, SP64 and SP50 were concerned, the concentrations chosen were seen to have an effect on platelet aggregation. Platelet inhibitors were dissolved in DMSO in a final percentage <0.3%,[24] DMSO was also added to the control samples, at the same concentration.

Filter aggregometer

We used the technique described by O'Brien and Salmon.[22] Tests were completed within 3 hours of the time of blood collection, an interval during which results have been seen to be reproducible. Five mL of whole blood, with or without inhibitors, were pushed through capillary-sized channels of a glass wool fiber filter at a constant pressure of 100 mm Hg and at room temperature. The glass fibers have a diameter between 0.1 and 3.4 µm, and the filter retains particles of 10 µm diameter or greater. Total red cell and leukocyte volumes were within normal limits in all individuals tested. Blood drops passing through the filter were collected after 50 and 100 seconds and then every 100 seconds for a maximum of 10 minutes. The blood was collected in tubes containing 10 µL of 100 mM EDTA. The number of blood drops passing through the filter was recorded by an automatic drop counter linked to a computer. The percentage of platelets and white cells retained in the filter was calculated on the basis of the pre- and post-filter counts which were performed with an automatic device (Counter T-890). Blood flow was recorded for a maximum of 10 minutes.

cAMP assay

Before starting the filteraggregometer test a sample of whole blood (0.5 mL), both under basal conditions and following treatment with different concentrations of the various adenosine derivatives, was used to assay for cAMP levels. The samples were treated with an equal volume of trichloroacetic acid (10%) to precipitate the proteins. They were then frozen and stored at –20°C. cAMP was measured using the cyclic AMP [^{3}H] assay system (Amersham). This assay is based on the competitive binding of cold cAMP to a high affinity protein for cAMP and a fixed quantity of [8–^{3}H] cAMP.

Platelet binding of an anti-P-selectin monoclonal antibody

To a sample of blood containing 10^6 platelets, 10 µL of CD41PE (this identifies integrin $\alpha_{IIb}\beta_3$) and 10 µL of CD62 (identifies P-selectin) conjugated with fluorescein isothiocyanate (FITC) were added. The final volume was brought to 100 µL with TBS buffer and the samples were incubated at room temperature for 15 min. Following this the samples were further diluted with 700 µL of TBS buffer. Platelet binding to the monoclonal antibody against P-selectin was measured with flow cytometry.[26]

Statistics

Means and standard deviations were calculated by an appropriate computer program and continuous variables were compared by means of Student's t-test for paired data.

Results

The effects of the compounds used were very clearly apparent, both on the dropping time and on the time required for all the blood to pass through the filter. Table 1 shows that all the compounds used to increase cAMP levels increased the blood flow through the filter. The baseline data of the 25 healthy volunteers used for this study did not differ from the historical data in our laboratory (after 50 sec=23.56±5.11, 100 sec=28.13±5.86, time required for all the blood to pass through filter 600 sec). The levels of cAMP in the blood were tested at baseline and after treating with PGE1, SP 64, 2-Cl-adenosine and NECA. Figure 1 shows the percent increase in platelet cAMP concentrations induced by the platelet inhibitors. PGE1 induced larger increases in cAMP than the other compounds. White blood cell retention in the filter test was evaluated after 50 and 100s. Figure 2 shows that the addition of each compound significantly altered the white blood cell retention compared to the retention in the control sample

Table 1. The effect of platelet inhibitors on blood passing through the filter.

	Number of drops passing through the filter		*Time required for all the blood to pass through the filter (sec)*
	50 sec	*100 sec*	
Basal	19.6±3.65	23±4.53	600
PGE_1 10 µM	45.8±1.3°	84.8±2.39°	95±7.07°
2-Cl-adenosine 20 µM	40.25±7.14°	74±11.66°	120±24.83°
NECA 1 µM	33.6±8.08°	59.6±4.53*	260±218.03°
SP 64 0.5 µM	32.2±6.14°	53±14.75°	171±48.53°
SP 50 0.1 µM	39.8±7.73°	69.4±18.45°	137±51.31°

*Mean ± S.D.; *=p<0.05, °=p<0.01 .*

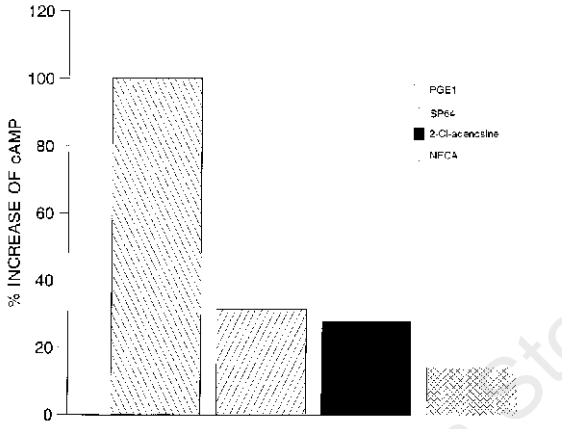

Figure 1. Increase of cAMP in treated samples expressed as % of baseline values for each compund.

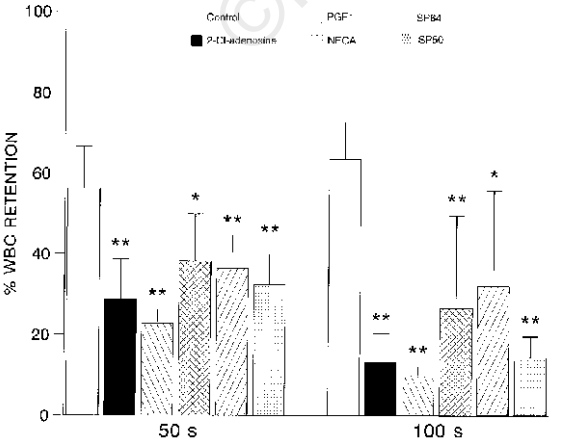

Figure 2. The retention of white blood cells is shown after 50 and 100 sec. *p<0.05; **p<0.01 vs. control.

(untreated). The percentages of white blood cell retained after 50 and 100s were: control 56.2±10.4, 63.4±9.23; 2-Cl-adenosine 28.5±10.25, 13.25±6.99; PGE1 22.7±3.46, 10±2.12; NECA 38.2±11.65, 26.6±22.83; SP64 36.2±8.32, 32±23.7; SP 50 32.4±9.61, 14.3±5.19, respectively. The data were analyzed after 50 and 100s, since it was during this period that most of the treated samples passed through the filter. The binding of a FITC-labeled monoclonal antibody against P-selectin was significantly reduced by the inhibitors after 100 sec. This is illustrated in Figure 3 (% of mean channel fluorescence at 100 s: basal 7.31±2.99; 2-Cl-adenosine 1.22±0.38; PGE1 1.02±0.11; NECA 2±0.99; SP64 1.54±0.43; SP 50 1.26±0.71). At all the time-points studied, platelet retention in the control and treated samples was not significantly different.

Discussion

Most of the papers published so far concerning the effects of shear stress in generating thrombi containing both platelets and leukocytes, have been performed in situations where platelets and leukocytes are separated and then mixed or using PRP. The big advantage of the filteraggregometer method is that whole blood can be used as such. Obviously, this method requires hirudin to be added as an anticoagulant, thereby thrombin is completely blocked and hence the effect of fibrin is not examined. We used adenosine derivatives, which increase the levels of cAMP and inhibit ADP-induced aggregation. Unlike adenosine these compounds are not internalized by platelets,[27] yet they retain the ability to stimulate adenylate cyclase by interacting with A_{2a} receptors.[28,29] From our data it would appear that there is no need to induce maximal levels of cAMP, since even when a

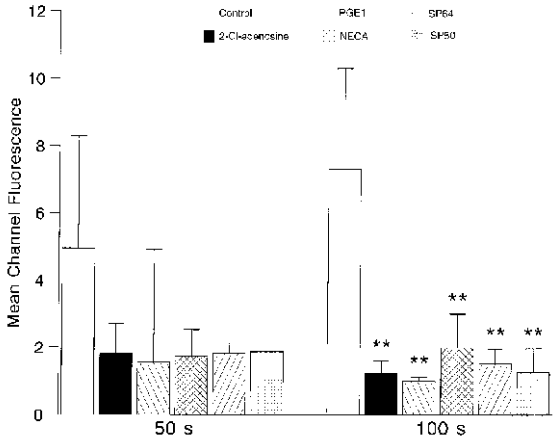

Figure 3. Platelet response after exposure to a FITC-labeled monoclonal antibody against P-selectin, expressed by mean channel fluorescence (MCF). *p<0.05; **p<0.01 vs. control.

small increase is present the observed effects still occur in the filteraggregometer. Data published so far have shown that prostaglandin I2 (PGI2) and prostaglandin E1 (PGE1) have important effects on the reduction of leukocyte numbers present in the thrombus formed. This has been associated with the platelets' inability to expose P-selectin.[14] The observed findings that blood treated with the compounds used in this study as well as PGE1, all known to increase the levels of cAMP, moves more freely through the filter and that more blood flows through the filter per unit of time can be considered a demonstration that platelets which stop inside the filter but do not bind leukocytes, give rise to a thrombus that does not occlude the filter pores. Using the filter aggregometer we studied the blood from particular clinical situations in which the number of white blood cells were elevated but the platelets were low. We observed that the filter was completely blocked after 50 sec. This was probably due to the binding of leukocytes to platelets present in the filter and a big thrombus was formed (data not published). This is confirmed by our new findings of reduced exposure of P-selectin on the platelet membrane, as a consequence of inhibition of platelet activation.[30] Decreased P-selectin expression was paralleled by decreased white blood cell retention within the filter, as a consequence of reduced platelet-leukocyte interactions. In conclusion, the filteraggregometer method described by O'Brien and Salmon can be considered a useful, simple and reproducible system not only to diagnose congenital disorders of primary hemostasis,[31] but also to investigate the effects of inhibitors on platelet-induced thrombus formation. Since it evaluates platelet function in the presence of other blood cells under high shear-rate, it mimics the *in vivo* situation better than the Born aggregometer.[32]

Contributions and Acknowledgments

MM studied platelet aggregation at the filteraggregometer and wrote the paper; GC synthetized adenosine derivatives, LG did the study with cytofluorimetry; PMM revised the paper and FP designed the experiments. Our thanks to Prof. M.Cattaneo from the Institute of Internal Medicine, IRCCS Maggiore Hospital and University of Milan, Italy, for discussing the data.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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