Platelet aggregation and flow cytometry analysis of canine platelet rich plasma

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SUMMARY

The aim of this investigation was to evaluate the suitability of flow cytometry for studying platelet function and aggregation in dogs. To that purpose, the platelet antigen P-selectin (CD62P) expression was monitored by flow cytometry and platelet aggregation by a turbidimetric method during an activation process induced by increased adenosine-5'-diphosphate (ADP) doses (2.5, 7.5 and 10 µM). Platelet aggregation was ADP dose-dependent and the flow cytometry analysis revealed a significantly increase of platelet membrane P-selectin expression according to time and to ADP doses reaching a threshold around 180-300 seconds for the 2 higher ADP doses (7.5 and 10 µM). In parallel, activated and micro-aggregated platelet populations were enhanced as revealed by morphological criteria. Consequently, the flow cytometry analysis allows detection of the early stages of platelet aggregation in dogs.

Keywords: Dog, platelet, flow cytometry, platelet aggregation, P selectin.

RÉSUMÉ

Agrégation plaquettaire et cytométrie en flux du plasma riche en plaquettes chez le chien

Le but de cette étude était d’évaluer la faisabilité d’une analyse par cytométrie en flux pour étudier les fonctions et l’agrégation des plaquettes chez le chien. Pour cela, l’expression de la P-sélectine (CD62P) en tant qu’antigène plaquettaire a été analysée par cytométrie en flux et l’agrégation plaquettaire a été mesurée par une méthode turbidimétrique durant l’activation des plaquettes par des doses croissantes (2.5, 7.5 et 10 µM) d’adénosine 5’ diphosphate (ADP). L’agrégation plaquettaire a été proportionnelle à la dose utilisée d’ADP et la cytométrie de flux a révélé que l’expression membranaire de la P-sélectine par les plaquettes augmentait significativement en fonction du temps d’exposition et de la dose d’ADP pour atteindre un plateau au bout de 180 – 300 secondes avec les 2 plus fortes doses d’ADP (7.5 et 10 µM). En parallèle, les critères d’analyse morphologique ont permis d’observer un accroissement de la population de plaquettes activées et/ou sous forme de micro agrégats. Par conséquent, l’analyse par cytométrie de flux permet effectivement la détection des stades précoces de l’agrégation plaquettaire chez le chien.

Mots-clés : Chien, plaquette, cytométrie en flux, agrégation plaquettaire, P-sélectine.

Introduction

Canine platelet activation has been associated with many cardiovascular, infectious, metabolic and auto-immune disorders [2, 14]. The activation process is often associated with several changes such as: reorganisation of the glycoprotein IIb-IIIa complex (GPIIb-IIIa) resulting in expression of a ligand binding function, granule membrane fusion with plasma membranes and platelet membrane binding of soluble proteins.

Few methods are commonly used to detect platelet activation either to analyse platelet morphologic modifications or to verify signalling pathways during activation. Flow cytometry is a sensitive and rapid research tool for the quantitative assessment of surface antigen expression on platelets. A relevant platelet antigen is P-selectin (CD62P), also known as GMP140, a glycoprotein belonging to a member of the C-type lectin family which is rapidly translocated from α-granules of platelets to the cell surface on stimulation [1]. CD62P also mediates the interaction between activated platelets and leukocytes which enhances the thrombus propagation, activates cytokine production in monocytes and may contribute to reperfusion injury [3, 7].

During platelet aggregation, the complex GPIIb/IIIa undergoes activation-dependent conformational changes and becomes competent to bind soluble fibrinogen. Then, fibrinogen cross-links platelets by bringing GPIIb/IIIa between adjacent platelets, leading to the formation of platelet aggregates [5]. Subsequently, there is a progressive stabilisation of platelet-fibrinogen interactions. These post-fibrinogen binding events are influenced by the extent of platelet secretion [11].

The CD62P antigen in canine platelets has been biochemically characterized [15] and its expression on activated canine platelets has been recently evaluated [10]. Nevertheless, the relationships between the aggregation process, the expression of CD62P and the modification of its expression during platelet activation were lacking and the authors suggested that further studies were needed to assess the functional role of CD62P. Consequently, the aim of this study was to monitor ADP induced platelet aggregation and P-selectin expression by flow cytometry.
Materials and Methods

The research was carried out on 10 healthy dogs of both sexes aged 1-5 years and belonging to different breeds (Table I). Ten millilitres of blood were collected from the jugular vein and immediately transferred into tubes containing sodium citrate solution (3.8%). Platelet rich plasma (PRP) was prepared by centrifuging whole blood at 180 g for 15 min at 20°C. PRP aliquots were prepared for each single test.

Platelet aggregation was assayed on PRP by using ADP as agonist by the turbidimetric method [8].

Flow cytometry analysis of the platelet surface antigen CD62P was performed by an indirect method using an anti-CD62P rabbit antibody followed by a fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit IgG antiserum. Preliminary studies defined the optimal amounts of each antibody (50 µg/ml and 10 µg/ml respectively) that would provide maximum antigen labelling and minimal unspecific binding. In activation experiments, a single dose of ADP was added to 50 µl of platelet rich plasma (PRP) at 37°C. Platelets were washed once by addition of at least 200-fold excess of fluorescence-activated cell sorting (FACS) buffer (Dulbecco phosphate-buffered saline, 2% fetal calf serum, 0.01% glycine). After centrifugation at 1500 g for 15 min, platelets were resuspended in 150 µl of FACS buffer. In order to identify CD61 expression (a β subunit of platelet GPIIb/IIIa constitutively expressed on platelets) 50 µl of platelet suspension, previously stained with CD62P according to indirect procedure, were incubated for 20 min at room temperature with 5 µl of monoclonal anti-CD61 antibody (dilution 1:10) or with negative control (IgG1 isotype-matched control). Platelets were analysed using a flow cytometer equipped with a 488-nm argon laser interfaced with a G3 Macintosh computer. The software utilised were Cell Quest and Paint-a-gate (Becton Dickinson). Platelet population was displayed as log forward-angle versus log side-angle light scatter plots. CD61 positivity was also required to define platelet population. A gate was set so that >95% of the platelets were included. Platelets, aggregates and micro-particles were distinguished from each other also by their forward scatter (FSC) profiles. All determinations were performed within 3 h of blood sampling.

For each experiment, at least three separate experiments were conducted. All experimental values are displayed as mean ± standard deviation. Statistical significance was evaluated by the Student t test and differences were considered as significant when p value was less than 0.05.

Table I: Epidemiological criteria of the studied dogs (n = 10).

<table>
<thead>
<tr>
<th>Dog (identification)</th>
<th>Breed</th>
<th>Sex</th>
<th>Age (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Golden Retriever</td>
<td>Male</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Mixed Breed</td>
<td>Male</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Boxer</td>
<td>Female</td>
<td>2</td>
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<tr>
<td>4</td>
<td>Boxer</td>
<td>Male</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Golden Retriever</td>
<td>Male</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Rottweiler</td>
<td>Male</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Mixed Breed</td>
<td>Female</td>
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<td>8</td>
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<td>Female</td>
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<tr>
<td>9</td>
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<td>Female</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>Mixed Breed</td>
<td>Female</td>
<td>5</td>
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Results

The results obtained by the turbidimetric method aggregation showed a reactivity of canine platelets proportional to the ADP as platelet activator (Figure 1). A low platelet aggregation (36.33%) was observed with 2.5 µmol/L of ADP whereas higher doses of ADP (7.5 and 10 µmol/L) induced stronger responses (66.7% and 92.89% of aggregation respectively).

The flow cytometry analysis of platelet CD62P expression was performed and expressed as mean fluorescence intensity (MFI) according to times (30 to 600 seconds) revealed that the protein expression was markedly modified by the added doses of ADP (2.5, 7.5 and 10 µmol/L) (Figure 2); whereas the fluorescence intensity remained low and slowly gradually increased from 30 sec. to 600 sec. with 2.5 µmol/L ADP, the higher ADP dosages induced marked and significant increases of P selectin expression compared to the previous ADP dose (p < 0.01) since the first 30 seconds until 600 seconds. The MFI strongly enhanced at the beginning of ADP stimulation for reaching a plateau since the 180th second with the highest dose (10 µmol/L). Furthermore, the CD62P expression rose more rapidly when platelets were activated by 10 µmol/L ADP instead of 7.5 µmol/L ADP (p < 0.01). Characteristic flow cytometry profiles based on the cell size (forward scatter - FSC) and cell granularity (side scatter - SSC) parameters during platelet activation are shown in Figure 3. Compared to the resting conditions (Figure 3A), the R1 area corresponding to the population of inactivated platelets became less intense 180 seconds after ADP (7.5 and 10 µmol/L) addition (Figures 3B and 3C) while a platelet sub-population containing aggregates was located in the R2 area. The size of the activated aggregated platelet group seemed to be proportional to the agonist doses.
Discussion

In the present study, as shown with the turbidimetric method aggregation, ADP can be considered as a platelet activator. In fact, this compound is well known as a mild agonist for platelet aggregation and its activity is due to the integrin receptor GPIIb/IIIa, whose activation induces changes in cell morphology and function and triggers a biochemical cascade which results in an aggregating effect [4].

This possibility of canine platelet aggregation induced by ADP was confirmed by the flow cytometry analysis of CD62P expression during ADP-induced platelet activation. This method allows a deeper analysis of the mechanisms involved in platelet aggregation. The platelet CD62P expression was not greatly modify by the 2.5 μM ADP dosage, probably because this low dose was not sufficient for inducing TXA2 formation and granule secretion, both needed for CD62P expression [15]. Considering the biological similarities of CD62P between canine and human platelets [15], the time-dependent increase of CD62P expression up to a maximum after 600 seconds induced by higher ADP doses (7.5 and 10 μM) was in agreement with similar studies on human platelets [9].

MERTEN and THIAGARAJAN hypothesised a specific sequence of events during aggregation [9]. The first event comprises the binding of fibrinogen with the active integrin receptor GPIIb/IIIa to allow a closer binding with platelets. As a result, CD62P comes to be expressed on platelet surface and binds more closely to platelets by the lectin domain thus providing stability to cell interactions and leading to the formation of large and stable aggregates. In this way, CD62P would not only be involved in platelet interactions with endothelial cells and leukocytes but also between platelets themselves [9]. In particular, it has been shown that the CD62P-carbohydrate binding has a high tensile strength and this may be a major mechanism in the stabilisation of platelet aggregation. After the formation of increasing numbers of these bonds over time, the initial GPIIb-IIIa –fibrinogen complexes can be internalised or moved toward the canalicular system, thus leaving CD62P as the only supply molecule. In the present study, platelet activation with 10 μM ADP significantly induced a more intense membrane P selectin expression during the first 180 seconds than with 7.5 μM ADP then lead to a fluorescence plateau whose the intensity
was similar with the 2 high ADP dosages. Consequently, according to the model of MERTEN and THIAGARAJAN, increased doses of ADP would favour the activation of the integrin receptor GPIIb/IIIa leading to the acceleration of the membrane CD62P translocation and not the synthesis of P selectin molecules de novo.

Other authors have suggested that CD62P participates in the aggregation process by different agonists and its involvement seems to be related to its association with the platelet cytoskeleton [13].

On the other hand, our results support that flow cytometry may be a useful tool for the study of morphological changes of canine platelets by exploring parameters other than the CD62P expression, which are important for the detection of prethrombotic states. The ADP-induced CD62P expression was positively correlated with platelet morphological changes. As seen in the dot plot analysis, the movement of the cloud on the right side suggested an increase of platelets which are morphologically more complex and an increase of CD62P expression was detected at the same time. Our analysis suggested a dose-dependent increase of platelets micro-aggregates which can be seen as shift of dots from the region of single platelets to higher levels of the side scatter (SSC) (R2 area). It is known that the presence of micro-aggregates is due to the Ca\(^{2+}\)-dependent binding of the coagulation factors Xa and Va on membrane to sites enriched with phospholipids that leads to an external migration of phosphatidyl-serine through the membrane bilayer [12].

As a conclusion, the flow cytometry analysis revealed a higher sensitivity with respect to conventional aggregometry in detecting the early stages of canine platelet aggregation (incipient aggregation) as already suggested for humans [6].

References


