The Effect of Paraprotein on Platelet Aggregation

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Background: Some patients with paraproteinemia have platelet aggregation disorders and the aim of this study was to examine disturbance of platelet aggregation in healthy blood donors by isolated paraprotein in vitro. *Methods:* Using Rivanol, paraprotein was separated from the serum of ten patients with paraproteinemia, who had decreased platelet aggregation with several inducers. Platelet aggregation in ten healthy donors was measured with and without addition of the isolated induced paraprotein. The test was repeated with added human immunoglobulins for intravenous use. *Results:* Average of maximal levels of platelet aggregation has been significantly decreased in plasma rich in platelets (PRP) of healthy donors after addition of paraprotein when inducers are used: adenosine diphosphate (ADP) ($P = 0.007$), collagen (COL) ($P = 0.008$), risto-cetin (RIS) ($P = 0.001$), and epinephrine (EPI) ($P = 0.002$). Average of latent time of platelet aggregation was significantly prolonged in healthy donors after addition of paraprotein with inducers: COL ($P = 0.008$), RIS ($P = 0.008$) and EPI ($P = 0.006$) while addition of human immunoglobulins caused no change in platelet aggregation. In comparison, when human immunoglobulins were added, maximal platelet aggregation and latent time did not change significantly. Paraprotein isolated from patients with paraproteinamia, who had decrease platelet aggregation, had significantly decreased platelet aggregation when added to PRP of healthy donors, in vitro. *Conclusion:* Platelet aggregation was not significantly changed was confirmed with addition of human immunoglobulins. J. Clin. Lab. Anal. 28:141–146, 2014. © 2014 Wiley Periodicals, Inc.

Key words: paraproteinemia; platelet aggregation; role of paraprotein

INTRODUCTION

Paraproteins are immunoglobulins synthesized and secreted by neoplastically transformed B-cell lines (lymphocytes and plasma cells) (1). Paraproteins are also known as M-component, which refers to malignant gammaglobulinemia in myeloma and macroglobulinemia (2). Since they are products of uncontrolled cell proliferation, their blood level is very high and they are detected as electrophoretically homogenous fractions, which can be distinguished from normal serum immunoglobulins (3).

Paraproteins with normal immunoglobulin structure are macromolecules consisting of four polypeptide chains, two of which are short (light (L) chains) and two are long (heavy (H) chains) (4, 5). Immunoglobulins have a 3D space structure characterized by the presence of globular unit domains. These are formed by specific wrapping of consecutive homologous segments, composed of 110 amino acids and interconnected by intrachain disulfide bonds (6). Each light chain has two globular domains and each heavy chain has four or five globular domains (7). The basic functional unit is an entirely symmetric globular molecule composed of 12 or 14 domains (6, 7).

The immunoglobulins are divided into five classes on the basis of differences between heavy chain domains. The five classes are immunoglobulins M (IgM) with μ heavy chains, immunoglobulins G (IgG) with γ heavy chains, immunoglobulins A (IgA) with α heavy chains, immunoglobulins E (IgE) with ε heavy chains, and immunoglobulins D (IgD) with δ heavy chains. IgG consists of four subclasses

or isotypes (IgG1, IgG2, IgG3, IgG4), and IgA consists of two subclasses (IgA1, IgA2). There are two types of light chains: kappa (κ) and lambda (λ). Each immunoglobulin isotype has both κ and λ chains, but a single molecule can be either κ or λ. Approximately two-thirds of the immunoglobulins have κ-type domains (κ/λ ratio— 2:1) (8, 9).

Paraproteins appear as molecules missing certain polypeptide chains, or their parts, as well as hybrid molecules with a variety of oligosaccharide chains, or as molecules with subtle changes without visible effects on the global structure. Structural alterations occur in both light and heavy chains of the paraproteins, as a consequence of genetic (deletions, insertions, conversions, point mutations) or posttranslational changes (10–14).

The most important physicochemical property of paraproteins is electrophoretic homogeneity, which is due to the identical electrical charge of the molecules enormously produced by an uncontrolled proliferating clone. The electrophoresis graphic record registers the paraprotein as a particular peak, the sharpness of which reflects clonality, while the intensity reflects the concentration. During electrophoresis, paraproteins move as a compact fraction, depending on the isotype variant of the immunoglobulin. IgG and IgM paraproteins are most commonly localized in the gamma, and IgA in the β electrophoretic field (15, 16). The carbohydrate component, interconnection of molecules into polymers, solubility etc. influence the electrophoretic homogeneity and rate of movement of paraproteins. Differences in the structure of the variable regions, by which the subclasses of light chains are determined, may lead to different electrophoretic mobility of paraproteins with the same type of light and heavy chains as well (3, 17).

Paraproteins often react nonspecifically with other plasma proteins: albumin, haptoglobin, α_1-glycoproteins, complement system proteins, coagulation factors and they can bind nonspecifically to various platelet receptors. The following are the most common hemostatic disorders— prolonged bleeding time, decreased platelet adhesion, impaired platelet aggregation, prolonged prothrombotic time (PT) and partial thromboplastin time (PTT), low concentrations of factors V, VII, VIII, and X, prothrombin, fibrinogen, and total protein. Moreover, paraprotein can inhibit factor VIII and vWF, or act as a lupus anticoagulant (3, 18–21). The aim of this research was to examine the role of paraprotein in platelet aggregation disorder in patients with paraproteinemia.

PATIENTS AND METHODS

Paraprotein was isolated from ten nontreated patients with paraproteinemia, nine of them with the diagnosis of multiple myeloma, while one patient had monoclonal gammopathy of undetermined significance (MGUS). Platelet aggregation disorder was established for all ten patients. Thus, platelet aggregation was decreased with all inducers used, but each patient had a normal platelet count.

Five microliter blood samples from each patient were allowed to clot, and after centrifugation the separated serum was frozen at $-70°C$. Paraproteins were extracted from the sera, which belonged to the IgG class. Platelet aggregation tests in plasma from healthy donors were performed in vitro with and without added isolated paraprotein. In order to exclude the possibility of nonspecific binding of paraproteins to the platelet receptors in vitro, the platelet aggregation test was repeated with added immunoglobulins for intravenous application.

Isolation of the Paraproteins

IgG paraproteins were partially fractionated from the serum samples by the Rivanol method. Rivanol solution (1.5%) was added to serum in a 1:1 volume ratio. The mixture was left overnight at 4°C, and afterwards centrifuged for 10 min at 3,000 rotations per minute (rpm). The supernatant contained IgG (so-called Rivanol-soluble IgG) and the protein concentration was determined by the tannin turbidimetric method. The relative abundance of protein fractions in the samples was determined by densitometry of electrophoretically separated fractions using Image Quant software (San Diego, CA). A horizontal electrophoresis device was used to fractionate IgG in 1% agar gel in an electric field of 8 mA for 30–40 min. The protein fractions were visualized by staining with amido black. The subclass of the isolated IgG paraproteins was determined by the Western blot method. After electrophoretic separation on agar gel, the proteins were transferred to a nitrocellulose membrane and allowed to dry in the air. All membranes were then incubated with mouse monoclonal antibodies specific for γ_1, γ_2, γ_3, and γ_4 heavy chains of human IgG. The characteristics of these monoclonal antibodies were defined in a collaborative study of the World Health Organization. After reaction with secondary antibody (goat antibody to mouse IgG) the spots were visualized with peroxidase using chloronaphthol as the substrate, which, in the presence of H_2O_2, gives a blue-purple precipitate (15, 22, 23).

Platelet Aggregation

Platelet aggregation was measured by Born's optical method, using whole blood aggregometer model 560. Venous blood was collected in a test tube containing anticoagulant sodium citrate in the ratio 1:9. Plasma rich in platelets (PRP) was obtained by centrifugation at 800 rpm for 15 min at 20°C, while plasma poor in platelets

(PPP) was obtained by centrifugation at 3,000 rpm for 15 min. Light ray transience through PRP is 100%, but through PPP it is 0% due to differing optical density of the samples caused by the presence of many platelets in PRP. After adding an agonist for aggregation, the optical density decreases while light transmittance increases. The signal occurring during aggregation converts automatically into an electrical impulse, which is transmitted to the printer. A graphic record of aggregation is thus obtained. PRP (450 μl) was poured into plastic tubes in the presence of a magnetic stick. Platelet aggregation was determined by adding 50 μl of agonist in the following concentrations: adenosine diphosphate (ADP) 10 μmol/l, collagen (COL) 10 μmol/l, ristocetin (RIS) 1.25 mg/ml, and epinephrine (EPI) 10 μmol/l. Aggregation monitoring time was 10 min. The maximal level of aggregation, expressed in transmission unit percentage, was determined from the graphic record of the aggregation curve. Normal values of aggregation are 60–100% (0.6–1.0). The latent period from the moment of adding the agonist till the beginning of aggregation was measured as well, where 1 sec corresponded to 1 mm on the aggregation graphic record. Cross-match between healthy control platelets and isolated paraprotein and initially disturbed platelet aggregation was performed as follows: First, 50 μl of 0.9% NaCl was added to 450 μl PRP from a healthy examinee in a plastic tube. The latent period until the beginning of aggregation and the final level of aggregation were measured after incubation at 37°C. Subsequently, 50 μl of paraprotein solution isolated from a patient with paraproteinemia and platelet aggregation disorder was added to 450 μl PRP from the same healthy donor and aggregation measured in the same way. The platelet aggregation tests were again repeated with addition of 50 μl of immunoglobulin for intravenous application to 450 μl PRP from the ten healthy donors instead of paraprotein (24).

Statistical Analyses

The statistical significance of differences was determined by Student's t-test for paired samples. The mean value (x) of the maximal level of platelet aggregation for all examined aggregation inductors was calculated for the healthy subjects after adding 0.9% NaCl to plasma. The average value (X) of the maximal level of platelet aggregation was also calculated after adding paraprotein to successive samples of each examinee's plasma for all examined aggregation inductors and the average change (y) was determined. For all obtained parameters, the t-test value (t) was calculated for the defined degree of freedom (df) and P-value (P) for the average value of maximal level of platelet aggregation, as well as the latent period. Statistical significance was set at $P < 0.05$.

RESULTS

After adding isolated paraproteins to successive samples of plasma from healthy donors, the average value of the maximal level of platelet aggregation with ADP differed from the values of 0.9% NaCl controls, $t = 3.51$, df $= 9$, $P = 0.007$. In the presence of ADP, there was no statistically significant change in average value of platelet aggregation latent period in healthy donors between 0.9% NaCl and isolated paraprotein. Moreover, when intravenous immunoglobulin was present in healthy donors' plasma, there were no differences in average values of latent period or maximal achieved platelet aggregation with ADP. A comparison of the values of maximal level of platelet aggregation of PRP with ADP directly sensed from the graphic record of the aggregation curve between 0.9% NaCl and isolated paraproteins is shown in Figure 1.

After adding isolated paraproteins to plasma from healthy subjects, the change in the average value of maximal level of platelet aggregation with COL was
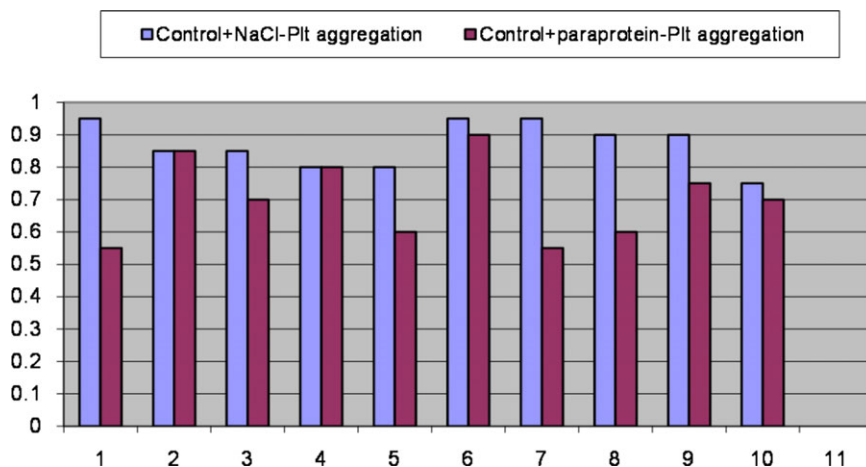

Fig. 1. Platelet aggregation by ADP in PRP of healthy donors before and after addition of paraprotein ($P = 0.007$).
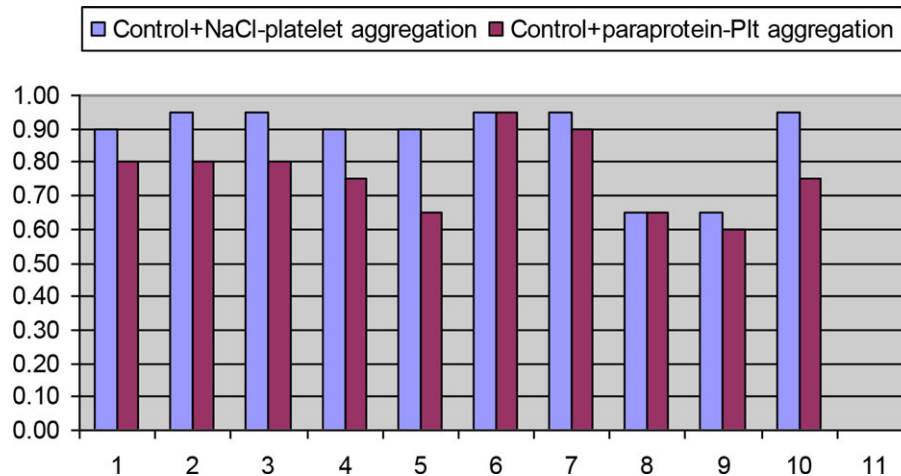
Fig. 2. Platelet aggregation by COL in PRP of healthy donors before and after addition of paraprotein ($P = 0.002$).

statistically significant, $t = 4.233$, df $= 9$, $P = 0.002$. These results are given in Figure 2. The average value of platelet aggregation latent period after adding paraproteins to plasma from healthy donors in the presence of COL was also statistically significant, $t = -3.417$, df $= 9$, $P = 0.008$. With intravenous immunoglobulins in the plasma from healthy donors, there were no significant changes either in average value of maximal level of aggregation, or in average value of the latent period.

In the presence of RIS, the change in the average value of maximal level of platelet aggregation after adding isolated paraproteins to plasma from healthy donors was statistically significant, $t = 4.599$, df $= 9$, $P = 0.001$ as shown in Figure 3. There was also a significant change in average value of platelet aggregation latent period in the presence of RIS, $t = -3.420$, df $= 9$, $P = 0.008$. After adding intravenous immunoglobulins to plasma from healthy donors, there were no significant changes in the average values of either maximal level of aggregation or latent period.

For EPI, the mean value of maximally achieved platelet aggregation changed significantly after adding paraproteins to plasma from healthy donors, in comparison to the average value obtained with 0.9% NaCl, $t = 4.382$, df $= 9$, $P = 0.002$. The comparison is shown in Figure 4. In addition, a significant change in the average value of platelet aggregation latent period with EPI also occurred, $t = -3.579$, df $= 9$, $P = 0.006$. After adding intravenous immunoglobulins, there were no significant differences either in mean value of maximal achieved aggregation with EPI, or in mean value of the latent period.

DISCUSSION

Paraproteins are structurally altered immunoglobulins synthesized by terminally differentiated B cells (lymphoplasmocytes and plasma cells) emerging by proliferation of a single clone (monoclone) (1, 9). Structural changes may occur both in heavy and light chains, as well as in the carbohydrate part of the paraprotein (8, 10, 11, 25).
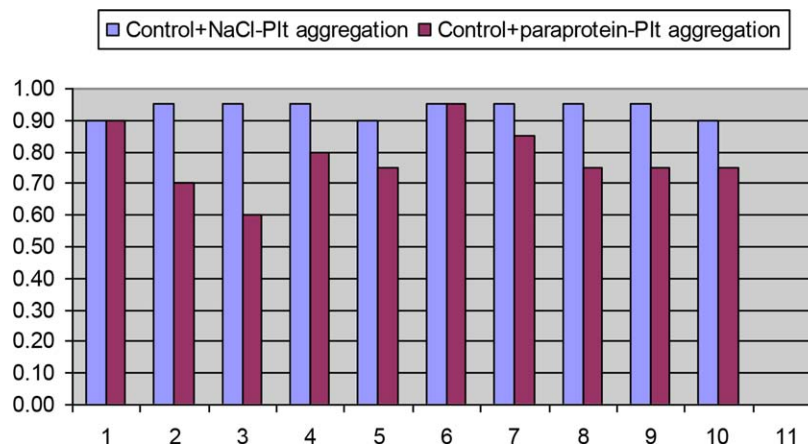

Fig. 3. Platelet aggregation by RIS in PRP of healthy donors before and after addition of paraprotein ($P = 0.001$).
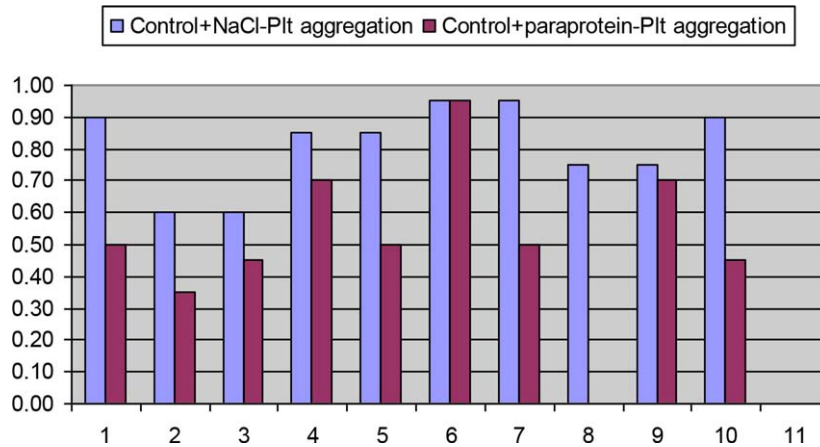
Fig. 4. Platelet aggregation by EPI in PRP of healthy donors before and after addition of paraprotein ($P = 0.002$).

The main roles of oligosaccharides in the structure of immunoglobins are determination of conformational stability, electric charge, and solubility of the molecule, and they are of key significance for achieving effector antibody functions, principally binding to complement and cell receptors. Moreover, they have an influence on affinity, elimination, and secretion of immunoglobulins (25).

Paraproteins can also react with a number of exogenous and endogenous antigens, as well as with nonbiological haptens. Most paraproteins bind to structures of their own organism (26, 27). Thus, paraproteins often show autoreactivity to Fc fragments of immunoglobulin G (rheumatoid factor activity), erythrocyte antigens (the activity of cold agglutinins), cytoskeletal proteins and DNA (polyreactive activity), and myelin glycoproteins. A paraprotein reacts with the supposed antigen throughout its Fab fragments in which antigen-binding spaces are localized (28).

The platelet function disorder occurs due to nonspecific binding of paraproteins to the platelet surface. In that way, receptor fields for different platelet aggregation agonists are blocked (19, 28). Among specific paraprotein targets, binding to the platelet glycoprotein IIIa, GPIb, as well as direct paraprotein binding to A1 domain vWF, has been described, which leads to disorders of platelet adhesion and aggregation (21, 29).

In order to assess the role of paraproteins in platelet aggregation disorder, we initially detected platelet aggregation disorder to the reagents ADP, COL, RIS, and EPI in a group of patients. Before implementing therapy, we froze the samples for isolation of paraproteins. First, platelet aggregation with above-mentioned aggregation inductors was performed in ten blood donors as healthy control subjects. The maximal achieved aggregation curve elevation and latent period until the beginning of aggregation were measured. Then paraprotein was added and the maximal achieved aggregation curve elevation and latent period were determined again. Isolated paraprotein from each patient had its own healthy control. Moreover, in contrast to normal immunoglobulins, excluding the possibility of nonspecific binding of paraproteins to platelet receptors in vitro and demonstrating that structural changes in paraproteins are the cause of platelet aggregation disorder; the maximal achieved platelet aggregation and latent period with ADP, COL, RIS, and EPI were determined for each healthy donor before and after adding immunoglobulin for intravenous application. In the presence of ADP, COL, RIS, and EPI significant changes were found in the average value of maximal achieved platelet aggregation after adding paraproteins to the plasma of healthy donors in vitro. Also, there were significant alterations in the average value of platelet aggregation latent period with COL, RIS, and EPI, but not with ADP. In contrast to this, no significant changes were found in the average values of maximal achieved platelet aggregation or platelet aggregation latent period with any of the tested aggregation inductors after adding immunoglobulins for intravenous application to plasma from healthy donors in vitro.

This research has indirectly shown that paraprotein, as a structurally aberrant immunoglobulin, may be the cause of platelet aggregation disorder in patients with paraproteinemia. Its mechanism of activity is probably binding to platelet receptors that participate in platelet aggregation. This leads to receptor block and the impossibility of aggregation agonists to bind to their specific receptors on platelets.

CONFLICT OF INTEREST

The authors have nothing to disclose.

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