Identification of a new potential mechanism responsible for severe bleeding in myeloma: immunoglobulins bind the heparin binding domain of antithrombin activating this endogenous anticoagulant

Bleeding is a relatively common complication in patients with multiple myeloma. Different factors have been involved, such as heparin-like anticoagulants, although the underlying mechanism remains obscure. The identification of a patient with a quiescent multiple myeloma IgG-γ that suffered a severe bleeding event controlled by rFVIIa and had prolonged thrombin time corrected by protamine sulfate gave the opportunity to evaluate whether antithrombin might be the target of these heparin-like anticoagulants. Antithrombin binding proteins from the patient were purified by fast liquid chromatography. The main antithrombin-bound protein of this patient was the paraprotein as demonstrated by Western blot and proteomic analysis. Intrinsic fluorescence and thrombin inhibition assays showed that the purified IgG of this patient bound antithrombin with K_D values in the picomolar range, activated antithrombin and increased the inhibition of thrombin as heparin. Interestingly, IgG purified from healthy controls also bound and activated antithrombin although with lower potency. We have identified a new element disturbing the thrombin-antithrombin axis. IgGs bind the heparin binding domain of antithrombin causing a similar activation to that provoked by heparin. This effect may have hemostatic consequences in patients with myeloma due to the high titer of paraprotein and potentially by differences in the IgG, which may contribute to the risk of bleeding of these patients.

The high titer of circulating monoclonal proteins present in patients with multiple myeloma and related plasma cell disorders are believed to play a relevant role in the hemostatic abnormalities frequently detected in these patients. The most common coagulation abnormalities in patients with plasma cell dyscrasias, prolonged thrombin time and reptilase time, are almost always asymptomatic and are explained by the monoclonal protein interference with fibrin clot formation.[1] Paraproteins may also target other hemostatic factors, such as platelet glycoprotein IIIa or FVIII, in all cases with severe bleeding consequences.[2] Thrombin has also been affected in several cases with multiple myeloma who suffered from severe bleeding through two mechanisms: direct inhibitors of thrombin and circulating heparin-like anticoagulants.[2-6] Although the pathophysiology of the hemostatic disorders caused by heparin-like anticoagulants remains obscure, both *in vitro* and *in vivo* treatment with protamine infusions have been effective.[1]

Since antithrombin is a key hemostatic element and the target of heparin, which acts as a co-factor leading to its conformational activation, we speculated that the paraprotein of patients with plasma cell disorders might also target this anticoagulant. This hypothesis was evaluated in a 73-year old woman with monoclonal gammopathy of undetermined significance who progressed to a quiescent multiple myeloma IgG-γ (2.5 g/L).

Since the diagnosis, the patient had had multiple bleeding events in arms and legs appearing spontaneously or after mild trauma. When the disease progressed, she spontaneously developed an extensive hematoma in the arm. The patient was treated with a dose of recombinant FVIIa (70 μg/Kg) and prednisone (20 mg/24 h), which controlled the bleeding. Then, six cycles of VMP (bortezomib, melphalan, and low dose of prednisone -60 mg) were administered, reaching only a partial response with mild reduction of the monoclonal component (1.7 g/L). No further bleeding events were reported.

Table 1. Thrombin time assay after *in vitro* addition of protamine sulfate to the plasma of the patient who suffered bleeding diathesis.

Protamine sulfate (mg/mL)	Thrombin time (seconds)
0	>600
10	187
50	76
100	22
200	21

Platelet function assay (PFA) studies, coagulation assays and analysis of coagulation factors were performed but only revealed a prolonged thrombin time (> 180s) and aPTT (75-97 sec, ratio 2.59-3.13) at all tested time points, including the moment of the severe hemorrhage and after treatment. Interestingly, the thrombin time was corrected by protamine *in vitro* (Table 1), and the reptilase time was always normal. The neutralization of heparin-induced bleeding by protamine sulfate[7] supports the presence of a molecule with a heparin-like effect in this patient. With the aim of identifying this factor, and to clarify the mechanism underlying the bleeding event of this patient, plasma proteins able to bind antithrombin were purified following the strategy shown in Figure 1A. This procedure revealed a main protein that was recognized by an anti-IgG polyclonal antibody. After a last protein purification step of anionic exchange, mass spectrometry proteomic analysis verified that the protein purified was an IgG isotype γ1. The same procedure was used in plasma from healthy subjects, rendering IgG of similar mobility than the control IgG (Figure 1B). Comparison of the electrophoretic mobility in SDS gels under reducing conditions revealed that the antithrombin-bound IgG purified from the patient had less mobility than control IgG (Figure 1B). No glycosylation abnormalities were detected in this protein (Figure 1C) and proteomic analysis of available peptides did not identify mutations or aberrant post-translational modifications (*data not shown*).

The anticoagulant effect of the IgG purified from the patient's plasma was assayed by evaluating thrombin and FXa inhibition. This IgG was able to activate antithrombin accelerating the interaction with thrombin and FXa slightly slower than unfractionated heparin or low molecular weight heparin, respectively (Figure 2A and B). Interestingly, although with reduced effect, control IgG also had anti-thrombin activity (Figure 2A and B). The acceleration of the inhibition of both FXa and thrombin suggests that the mechanism of inhibition happens by the allosteric activation of antithrombin, although the inhibition of thrombin is favored (Figure 2A). The acceleration of the inhibition of these targets is caused by the IgG since neutralization is provoked by protamine sulfate (Figure 2C). To confirm the activation of antithrombin suggested by the generation of thrombin-antithrombin or FXa-complexes, a study of the intrinsic fluorescence of antithrombin was performed. It is well known that antithrombin is activated after binding to heparin, which increases the emission of fluorescence when antithrombin is excitated at 280 nm.[8] Our results showed that the

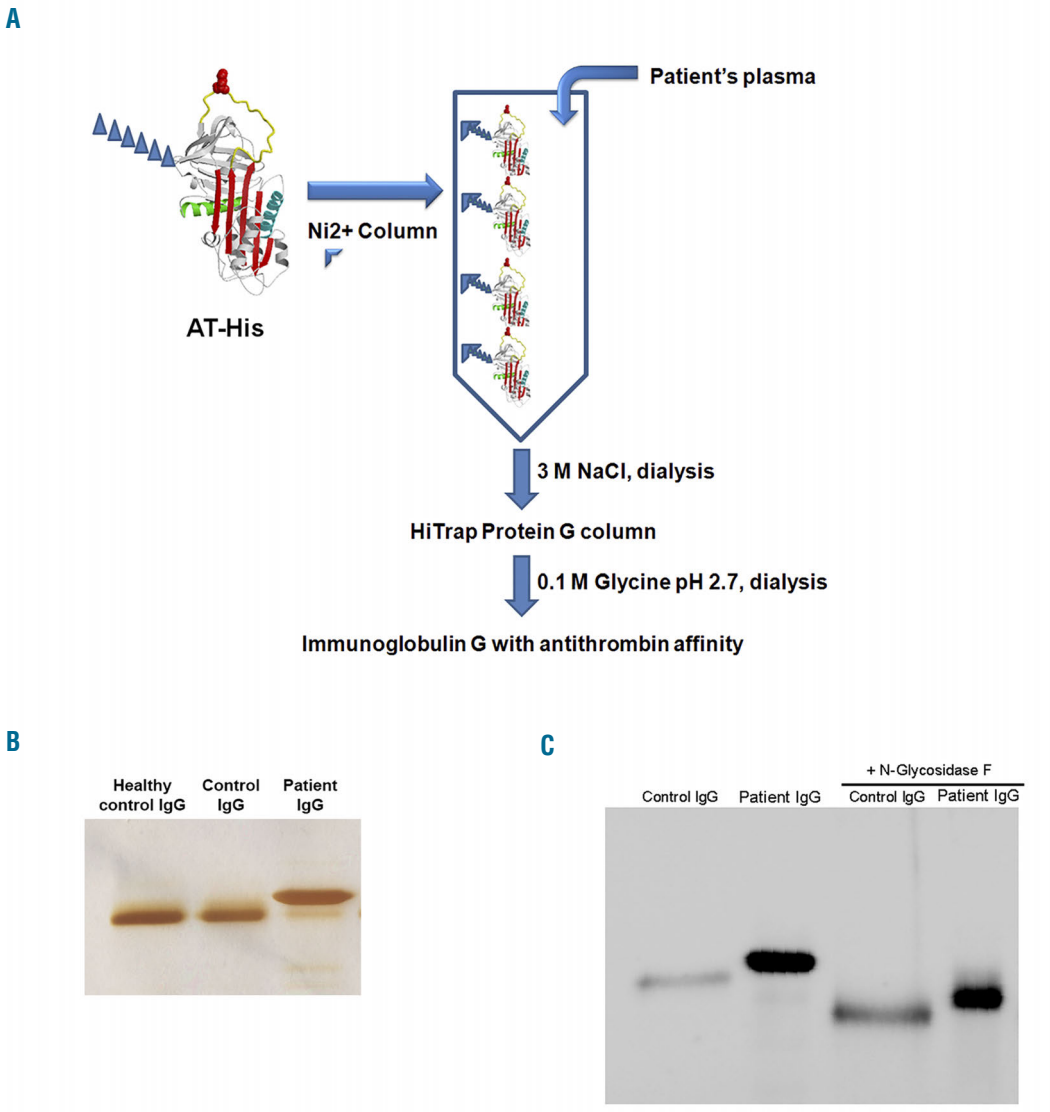

Figure 1. IgG purification. (A) Purification strategy of antithrombin-binding IgG from the plasma of the patient with multiple myeloma and healthy subjects. Recombinant wild-type antithrombin was generated with a 6-histidines tag at the C-terminal. Plasma from a patient with multiple myeloma who developed a severe coagulopathy and healthy controls were loaded into a Ni2+-histidine-antithrombin column. After extensive washing, those components in the plasma with ability to bind to antithrombin were eluted. After dialysis, eluted products were further purified using a HiTrap Protein G column. (B) Electrophoretic mobility (8% SDS-PAGE under reducing conditions) of purified antithrombin-binding IgGs from the patient with multiple myeloma who suffered bleeding diathesis and a healthy control. As control, IgG purified from healthy subjects (Flevogamma IV, Grifols) were also loaded (Control IgG). Detection of proteins was carried out by silver staining. (C) Western blot using human anti-IgG polyclonal antibody after treatment of purified proteins with N-Glycosidase F.

IgG component purified from the patient was able to activate antithrombin as heparin did (Figure 2C). This activation was achieved with a very low concentration, which indicated a high affinity interaction between both proteins. The change in intrinsic fluorescence of antithrombin (50 nM) upon titration of the IgG was monitored at 340 nm on a Cary Eclipse spectrofluorometer. The K_D value was in the picomolar range (23.7±3.8 pM), which is smaller than the K_D for the pentasaccharide (0.2±0.07 nM), although comparison of K_D values of a large protein (purified IgG) and an oligosaccharide (pentasaccharide) is difficult. According to functional assays, also control IgG and IgG purified with the same procedure from healthy subjects activated antithrombin, although the K_D value was approximately 400-fold higher than that observed for the IgG of the patient and pentasaccharide (14.5±2.1 nM for control IgG and 10.0±0.1 nM for antithrombin-bound IgG from healthy subjects). The IgG-mediated activation of antithrombin was also evaluated by measuring the intrinsic fluorescence emission upon titration on a variant antithrombin (Antithrombin Toyama, p.Arg79Cys) with reduced heparin affinity. As expected, addition of control IgG, the IgG purified from the patient, or heparin did not provoke any measurable fluoresce emission enhancement on this mutant (*data not shown*).

An exquisite control of thrombin is warranted in order to prevent excessive, spontaneous or mislocalized thrombin generation. The main endogenous inhibitor of thrombin is antithrombin. Thus, even minor modifications of the thrombin-antithrombin axis might have opposite pathological consequences. Thus, congenital antithrombin deficiency caused by mutations or deletions in *SERPINC1*, the gene encoding antithrombin, significantly increases the risk of primary and recurrent thrombosis.

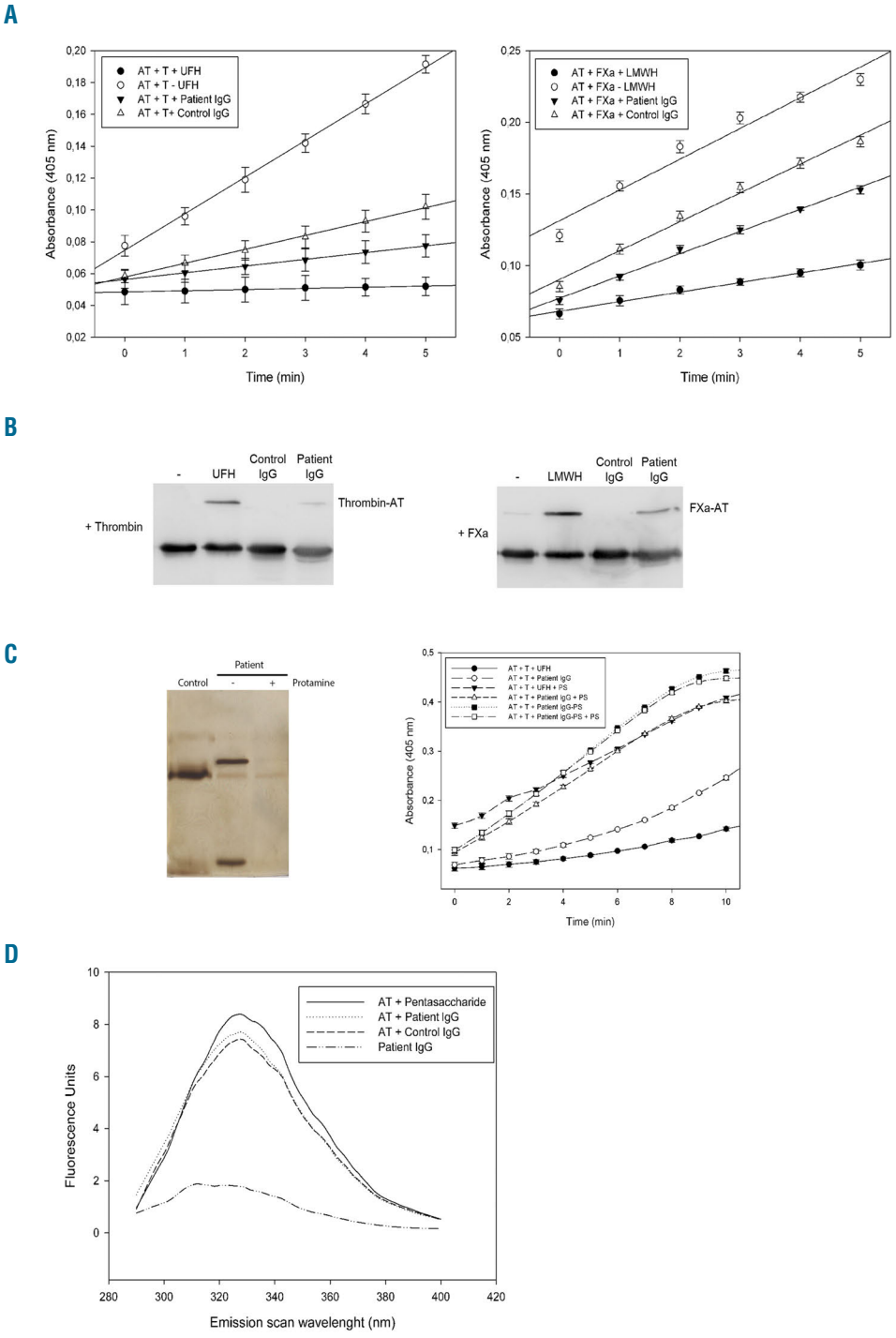

Figure 2. Activation of antithrombin by IgGs. (A) Thrombin and FXa inhibitory function of antithrombin in absence and presence of unfractionated heparin (UFH) or low molecular weight heparin (LMWH), control IgG or IgG purified from the patient with multiple myeloma (MM) who suffered bleeding diathesis. Thrombin and FXa activity were determined by a chromogenic substrate assay. The figure summarizes the results obtained from two independent experiments performed in triplicate. (B) Detection of thrombin-antithrombin (T-AT) and (FXa-AT) complexes by 8% SDS-PAGE under reducing conditions and Western blot with human anti-antithrombin polyclonal antibody, representative of two independent experiments. (C) Neutralization of IgG by protamine sulfate (PS). (Left panel) IgG purification from plasma of the patient. Plasma was loaded onto the Ni2+-antithrombin column directly or after incubation with PS (0.3 mg/mL). Samples were run in an 8% SDS-PAGE under reducing conditions and stained with silver. As control, IgG purified from healthy subjects (Flevogamma IV, Grifols) were also loaded (Control IgG). (Right panel) Anti-FIIa activity of AT and neutralization by PS. AT was incubated with unfractionated heparin (UFH), Patient IgG or Patient IgG purified from plasma incubated with PS (Patient IgG-PS). Neutralization of the activation of AT with PS was carried out by incubation with UFH, Patient IgG or Patient IgG-PS previously to the reaction with Thrombin. (D) Intrinsic fluorescence emission upon antithrombin activation. The increase in intrinsic fluorescence of antithrombin (50 nM) upon addition of the purified IgG from MM patient and control IgG (200 nM) was monitored from 290 to 340 nm on a PerkinElmer Life Sciences 50B spectrofluorometer, with excitation at 280 nm and using bandwidths of 2.5 nm for both excitation and emission. The experiment was carried out at room temperature under physiological ionic strength (I=0.15) in 20 mm Na2HP04, 100 mm NaCl, 0.1 mm EDTA, 0.1% polyethylene glycol 8000, pH 7.4. The same spectrum was also done with pentasaccharide (10μM) in order to establish a comparison. The intrinsic fluorescence of the IgG purified from the patient is also shown. Experiments were performed in duplicates.

Actually, congenital antithrombin deficiency was the first thrombophilic factor identified and so far has remained the strongest. Similarly, two mutations in the prothrombin gene disrupt the sodium-binding region, which is crucial for a correct interaction of thrombin with antithrombin. The variant prothrombins are highly resistant to antithrombin inhibition, ensuing a prolonged procoagulant activity and susceptibility to thrombosis.[9] On the other hand, a point mutation (p.Met358Arg) in *SERPINA1*, the gene encoding other serpin with no anticoagulant role, α1-antitrypsin, generates a variant (α1-antitrypsin Pittsburgh) with a greatly impaired anti-elastase activity but significantly increased antithrombin activity, which provoked bleeding disorders.[10] Finally, activation of antithrombin by different compounds acting as co-factors (including sulfated small organic ligands such as lignins and flavonoids, heparin and heparin-like molecules) significantly increase the risk of bleeding.[11]

The high reactivity of the heparin binding site of antithrombin allows the binding of different molecules through electrostatic interactions. However, not all interactions cause activation of antithrombin.[9,12] This study identifies a new molecule able to bind the heparin binding domain of antithrombin with functional consequences acting as a heparin-like factor. The IgG from a patient with multiple myeloma, who suffered from a severe bleeding event, had a heparin-like activity. The binding of this IgG to the heparin binding domain of antithrombin activates the serpin similarly to heparin, explaining the prolonged thrombin time and bleeding diathesis of this patient. Accordingly, protamine inhibits the effect of the paraprotein. Interestingly, our study also demonstrated that all IgG are able to bind antithrombin with milder activating functions. Therefore, increased levels of IgGs might potentially increase the risk of bleeding. It is possible that particular features of the monoclonal IgG might exacerbate this anticoagulant effect, as in the case of the patient suffering bleeding diathesis. Further studies are required to verify this hypothesis, and to find the potential differences that might have prognostic relevance in patients with myeloma and related plasma cell disorders.

Finally, we cannot forget that the interference on the thrombin-antithrombin axis may also affect the risk of thrombosis. The binding of certain IgGs to the heparin binding domain of antithrombin might impair the activation of antithrombin by physiological co-factors when it is required, thus also contributing to the risk of thrombosis, which is also a relatively frequent complication in these disorders.[13] Purification following our procedure and analysis of the IgG of patients with myeloma and related plasma cell disorders who develop thrombosis may help to answer this question.

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