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FORUM

Tissue factor pathway inhibitor revisited

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Early studies

Reading the paper by G. J. Broze in this journal [1] awakened long-dormant memories, some good, some less so. The latter because I did not succeed in isolating and characterizing tissue factor pathway inhibitor (TFPI), then anticonvertin. However, I had no chance with the methods available at that time. These were classic clotting methods, adsorptions, precipitations, dialysis, activation/inactivating studies, temperature tolerance, with no need for a spectrophotometer. There was also a joker in the pack: factor X, discovered simultaneously in Oxford and Chapel Hill in 1956, when I was completing my thesis.

Owren and others, referred to in the paper and by me, had noted that the thromboplastin and proconvertin (TF–FVII) complex rapidly lost activity in serum. As my work concerned intermediate reactions in blood on addition of tissue factor (TF), studying this phenomenon was a natural consequence. A necessary step was to 'purify' these, the only two protein components known to be involved at that time. After careful removal of blood, TF was homogenized in a mixmaster, centrifuged, resuspended and washed once. This supernatant was neutralized to 7.35, distributed in small aliquots diluted to maximal activity and frozen at – 20 °C. After thawing and being kept on ice, activity was stable. Exhaustive studies, including several washings, showed some contamination, but this were kept constant by the standardized procedure. To sediment TF, $1.6 \cdot 10^5$g was used; this gave empty supernatant. Rigorous procedures for substrate plasmas also provided acceptable reproducibility. For TF assay, one part platelet-poor activated citrated human plasma was mixed with two parts double Seitz-filtered ox plasma [to secure factor (F)V and fibrinogen level]. FV, necessarily present in the substrate plasmas both for test and control mixtures, was not a variable, and purification was unnecessary. The serum preparations applied were obtained by complete coagulation after withdrawal, then TF was added and further incubated. After high-speed centrifugation to remove convertin, oxalate was added, the mixture adsorbed three times with barium sulfate and carefully dialyzed. This was done to avoid oxalate in the test system that could reverse inactivation of the TF, and it was free of factor II and factor VII.

Purification has a quite different meaning today. However, even though many impurities were present in these reagents, the many control experiments indicated that they did not invalidate the results. An exception to this is the presence of FX.

With these reagents the behavior of the variables was studied in a variety of settings, in an attempt to shed light on their separate roles. First, it was shown that TF and FVII were stable in adsorbed serum, whereas convertin (the product of the two with Ca^{2+}) was progressively inactivated (Fig. 1 in the Broze paper). This left no doubt that only the combination of the two constituents was inactivated. Similar experiments were performed with serum from those with congenital FVII deficiency. As expected, TF was scarcely affected since practically no convertin could be formed, but preformed convertin was inactivated precisely as in normal serum. Thus, the inhibitor was present in normal amounts.

The possible influence of Ca^{2+} was studied. By adding citrate, oxalate or EDTA at the top of the inactivating curve, inhibition was immediately reversed with EDTA and oxalate, but only slowly with citrate. This indicated a strong Ca^{2+} binding in the complex. Further studies clearly established a quantitative relation between FVII and TF both in formation and in inactivation. Mixing increasing amounts of FVII with fixed amounts of TF, serum, or *vice versa*, with Ca^{2+}, a saturation point was always observed. This stochiometric relationship showed as a straight line between convertin concentrations and amount inactivated per unit time.

The physicochemical properties of the inactivator were then studied. It was heat labile, resistant to acid/alkali challenges, not adsorbable, not dialyzable, not affected by glass powder activation, and only half of its activity was recovered in ammonium sulfate precipitation. This is compatible with the inhibitor being a protein. It was unlikely to be an enzyme for different reasons, e.g. the constituents of the complex could be recovered undisturbed, and the straight-line relationships.

It is questionable whether I could have proceeded successfully if FX-deficient plasma had been available. It would then have been possible to have reagents with FVII and FX present separately, and thus the pivotal role of FX could have been established. However, in view of the sophisticated methodology needed, as evident from the race

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for the identification of TFPI, it is unlikely that I would have been successful at that time. What a gulf there is between these macrodimensions and present-day applied concentrations in the low picomolar range!

It is astonishing that so much time elapsed before anticonvertin became a cloned and fully characterized TFPI. It was no surprise that it was Sam Rapaport's laboratory that restarted the search – he was in Oslo during my studies.

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Reference

1 Broze GJ. Rediscovery and isolation of TFPI. *J Thromb Haemost* 2003; **1**: 1671.

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George Broze nicely summarized the history and key events that led to the rediscovery and isolation of tissue factor pathway inhibitor (TFPI) [1]. The term 'rediscovery' is related to the remarkable time span from the initial studies demonstrating the presence of an inhibitory principle in serum during the 1950s until the inhibitory mechanism was elucidated and the inhibitor purified during the 1980s. A major contribution in the field took place in Oslo under the leadership of Paul Owren, who had discovered blood coagulation factor V [2]. First, Knut Aas, in a doctoral thesis (1952) suggested the presence of 'anticonvertin' or factor (F)VIIa inhibitor in blood [3]. Second, Peter F. Hjort in his doctoral thesis (1957) provided convincing evidence of a slow-acting 'anticonvertin' in human serum, which appeared to form a stoichiometric complex with 'convertin', later known as the FVIIa–tissue factor (TF) complex [4]. Finally, Sam Rapaport received the first Fulbright scholarship to Norway to visit Owren's laboratory during 1953–1954 and formed a strong friendship with Hjort. This visit and friendship played an important role in the events that took place approximately 30 years later.

A further contribution to the field of coagulation inhibition of later importance was achieved during the 1960s when, in Owren's laboratory, Olav Egeberg reported inherited antithrombin deficiency causing thrombophilia [5]. Moreover, one of us, in a satellite laboratory established by Owren's students Knut Aas and Hans Christian Godal in Oslo, managed to purify antithrombin to homogeneity [6] and to demonstrate the interaction with heparin [7]. This led to a lasting interest in natural anticoagulants, anticoagulant therapy, and the role of heparins in particular. The old observations on an inhibitory principle of the extrinsic pathway remained a continuous puzzle and in 1978 an effort was made to develop an assay of the putative inhibitor. Purified FVII and TF were incubated with barium sulfate adsorbed plasma. After incubation, residual FVIIa–TF activity was determined by the sequential addition of factor (F)X and a chromogenic substrate. Furthermore, the inhibition was found to elute in three different peaks by gel filtration, which in retrospect corresponds to the elution pattern of lipoprotein fractions and free TFPI [8]. A major concern was that the inhibitor seemed to be lost upon multiple adsorptions of plasma.

We therefore read with great interest the report of Sanders and colleagues [9], initially reported as an abstract at the International Society on Thrombosis and Haemostasis meeting in San Diego in 1983, that FX was necessary for the inhibition of FVIIa–TF. This explained the previous loss of activity of the inhibitor upon barium adsorption [8] which also removed FX necessary to facilitate inhibition. We consequently constructed a new and reproducible assay for the quantification of TFPI in plasma samples, and confirmed that FXa was necessary for inhibition [10], as had been shown in Rapaport's [11] and Broze's [12] laboratories. In a remarkably short time the inhibitor was purified and cloned [13].

We utilized our new assay to explore the plasma levels of TFPI in clinical materials. Our hypothesis was that TFPI would be low in cases of severe TF-induced disseminated intravascular coagulation. It was therefore a great surprise when we found that severe sepsis was associated with elevated levels of TFPI in plasma [14,15]. We had also anticipated that vascular surgery would release TF and therefore consume part of the inhibitor. Again, our finding told us a different story. The highly elevated levels of TFPI in patients undergoing elective vascular surgery could later be explained when we learned that these patients had been given a bolus heparin injection during surgery [16]. Further studies demonstrated the ability of heparins to release TFPI from the vessel wall [17] and that heparin-releasible TFPI contributed significantly to the anticoagulant effect of heparins [18,19]. However, the physiological role of heparin-releasible TFPI remains controversial [20].

For us an interesting epilogue relating to the rediscovery of TFPI took place when one of us, scientifically spoken being the

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