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Cellular and Molecular Life Sciences

Memories of a Senior Scientist

Contributions to the evolution of knowledge about hereditary hemorrhagic disorders

H. R. Roberts

Sarah Graham Kenan Distinguished Professor of Medicine, Laboratory Medicine, and Pathology
University of North Carolina School of Medicine, Campus Box #7035, Chapel Hill, North Carolina 27599-7035 (USA), Fax +1 919 966 7639, e-mail: hrr@med.unc.edu

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I have often wondered why those of us in experimental medicine choose a particular field for study. In my case, I became interested in blood coagulation while a medical student at the University of North Carolina, and the main reason for my interest stemmed from the inspiration of a superb investigator and teacher, namely Professor Kenneth Brinkhous. He was one of the most exciting teachers that I have ever encountered, and his native inquisitiveness and enthusiasm about blood coagulation were contagious. Professor Brinkhous and many other colleagues at the University of North Carolina over the last half century were directly responsible for my interest in, and contributions to, the field of blood coagulation and the clinical disorders of hemorrhage and thrombosis.

One of my most memorable experiences began with our first purification of blood coagulation factor VIII, then known as the antihemophilic factor (AHF) in 1965. [1] Using the technique of Wagner, Dr. Philip Webster and I used the aliphatic amino acid glycine to precipitate factor VIII from human plasma to obtain a factor VIII fraction at least a hundredfold purified over the beginning product. Realizing that most of the factor VIII was in the cold insoluble part of plasma that remained after slowly thawing frozen plasma, we later used this cryoprecipitate as our starting material. The glycine-precipitated factor VIII products were sufficiently concentrated to permit us to completely normalize the factor VIII levels in patients with classic hemophilia without overloading the circulatory volume. This was the first time that hemophilic patients could have their factor VIII levels raised to normal. [2] Before factor VIII was concentrated, we only had normal plasma as a source of factor VIII, and it was not possible to obtain factor VIII levels above 15–20% without overloading the patient's circulation. After our initial experiments in humans, our factor VIII product was developed by a commercial firm, Hyland Laboratories, a part

of Baxter, to develop the first commercially-available factor VIII concentrate called Hemofil. [3] Dr. Edward Shanbrom, then Medical Director of Hyland Labs, was instrumental in obtaining approval of the product from the Food and Drug Administration.

The availability of lyophized, potent, and stable factor VIII concentrates revolutionized the treatment of patients with classic hemophilia. But commercial development had its drawbacks since the commercial product was prepared from starting pools of plasma sometimes collected from as many as 20,000 different donors. These large pools led to contamination of the lots of factor VIII with transmissible agents including hepatitis B and C viruses and eventually the retrovirus that resulted in the acquired immune deficiency syndrome. [4] These tragic events were not solved until around 1984–1985 when solvent-detergent and/or heat treatment methods were perfected sufficient to inactivate most (but not all) transmissible agents. Recombinant factor VIII is currently available and is unlikely to lead to a repeat of the above diseases. [5]

In the late 1960s we were also the first to show that elevated factor VIII was a risk factor for venous thrombosis. [6] This observation has recently been confirmed in experiments which show that an increased factor VIII level in some families seems to result in venous thrombosis. Why arterial thrombosis does not seem to be correlated with elevated factor VIII levels is not known. This older observation has been of particular interest to me in view of recent observations by others that lowering factor VIII levels using anti-factor VIII and IX antibodies can prevent both arterial and venous thrombosis, at least in experimental animals. [7] It is likely that lower factor VIII levels in humans are also protective against thrombosis since carriers of classic hemophilia with factor VIII levels of about 50% of normal are less prone to thrombotic complications than people with normal levels of the factor. [8]

Another memorable experience was our discovery of the first patient with the congenital deficiency of all the known vitamin K-dependent coagulation factors including blood clotting factors prothrombin, factors VII, IX, X, and proteins C and S. [9] This was discovered in an infant who bled extensively and who had no detectible prothrombin or other vitamin K-dependant coagulation factors. This patient responded to large doses of intravenous or oral vitamin K, even though she was not deficient in vitamin K. However, her response to vitamin K was not complete, but did result in elevation of the vitamin-K dependent factors to about 15 to 20% of normal, which was sufficient to keep her prothrombin time around 20 seconds, very similar to the therapeutic level expected after warfarin administration. We also described a second patient with a similar disorder and postulated that the cause of both disorders was a mutation either in the vitamin K-dependent carboxylase or vitamin K-dependent reductase. [10] Both the carboxylase and reductase enzymes have now been cloned and sequenced by our colleague, Dr. Darrell Stafford. [11, 12]

In 1968 we were the first to clearly distinguish genetic variants of hemophilia B due to factor IX deficiency. [13] Using a human anti-factor IX antibody, we showed that some severely affected patients with hemophilia B had no detectable factor IX antigen while other patients had either normal or reduced levels of factor IX protein albeit with decreased clotting activity, which varied from undetectable to very low levels. We referred to these variants as being cross-reactive material negative (CRM^-) or positive (CRM^+) or reduced (CRM^r). These studies clearly demonstrated the genetic heterogeneity of hemophilia B. The same technique was later used by others to show that classic hemophilia was also genetically heterogeneous. At the present time, of course, the genes and amino acid sequence of both factor VIII and factor IX are known, and there are several hundred distinct mutations of each protein.

Later, using affinity chromatographic techniques, we isolated factor IX protein from a CRM-positive hemophilia B patient and found that the defective molecule had a histidine at position 145 instead of an arginine (R145H substitution). [14] This was the first description of an abnormal factor IX molecule, which we named factor IX Chapel Hill. As a result of the mutation at position 145, factor IX Chapel Hill could not be activated by either the tissue factor/factor VIIa pathway or by factor XIa. Cleavage of the R145 bond was essential for release of the activation peptide, which converted the factor IX zymogen to an active enzyme, factor IXa beta. Interestingly, the activation peptide in factor IX Chapel Hill could be released by trypsin (3 amino acids proximal to the normal cleavage site) to result in an active enzyme. Subsequently, we and others characterized numerous mutants of factor IX correlating the results with the function of factor IX in its reactions with its activators, co-factors, and substrates.

In 1972 and 1973, I had the privilege of spending a sabbatical year with Professor Staffan Magnusson at the Molecular Biology Institute at the University of Aarhus in Denmark. My purpose in working with Professor Magnusson was to study the molecular biology of factor IX, but when I arrived in Aarhus, Magnusson asked if I would mind isolating and sequencing the anticoagulant from the leech, *Hirudo medicinalis*. He gave me a small black plastic vial containing a fluffy white powder which he had obtained from Dr. D. Bagby in Romania and said to contain purified hirudin. Actually, on SDS gel electropheresis, the material contained several proteins, but we were able to isolate the one batch that contained hirudin, a highly potent thrombin inhibitor. From this sample we were able to determine the amino acid sequence of the material. [16] Commercial development of hirudin analogs are now available as potent anticoagulants.

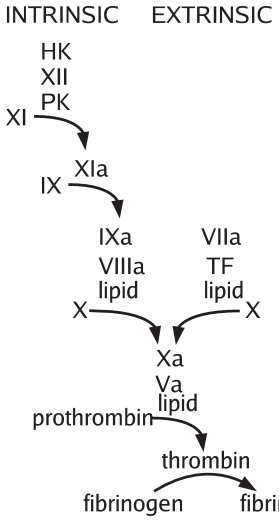

Figure 1. *Cascade model of coagulation*. This scheme reflects the components tested for in the prothrombin time assay (extrinsic pathway) and the activated partial thromboplastin time assay (intrinsic pathway). The intrinsic pathway consists of HK, factor XII, prekallikrein (PK), factor XI, factor IX, factor VIII, factor X, factor V, and prothrombin (II), which is converted to thrombin (IIa). The activated form of these factors is indicated by adding the letter 'a' as a suffix (eg, IXa). The extrinsic pathway consists of TF, factor VII, factor X, factor V, and II. Reactions requiring a phospholipid surface are indicated by lipid.

However, the most exciting and rewarding part of my career came later, beginning in the 1990s. My colleagues (Dr. Dougald Monroe and Dr. Maureanne Hoffman) and I had always been interested in the process of hemostasis, recognizing that the classic waterfall or cascade hypothesis was not adequate to explain the reactions leading to the formation of an impermeable hemostatic plug. Professors Davie and Ratnoff in the United States and Macfarlane in the United Kingdom had formulated the waterfall or cascade hypothesis of blood coagulation. Their experiments suggested that coagulation could be initiated by contact activation of factor XII as shown in Figure 1. [17, 18] They also commented on the potential role of tissue factor and platelets, though it remained for Professors Yale Nemerson and George Broze (among others) to clearly show that coagulation was initiated by tissue factor/factor VIIa. [19, 20] In our efforts to study this phenomenon, we reasoned that *in vivo*, cells containing tissue factor and activated platelets, had to play important roles in the formation of a stable hemostatic plug. Therefore, in an effort to mimic as closely as possible the *in vivo* conditions, we set up a cell-based coagulation assay using activated monocytes as a source of tissue factor and unactivated platelets as a surface for thrombin generation. This technique required that we use microtiter wells, where we could add both cells and plasma concentrations of all the procoagulants and naturally occurring inhibitors in order to study how cells and soluble clotting factors could in-

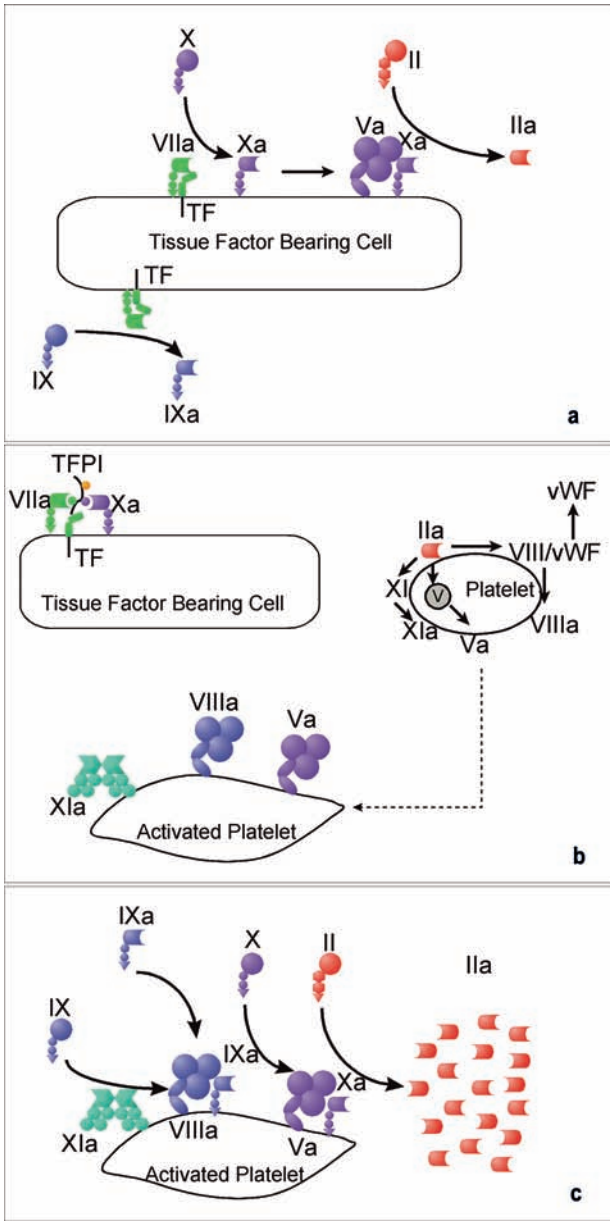

Figure 2. *Cell based model of coagulation*. In this scheme, coagulation occurs in three phases: initiation, priming, and propagation. In the initiation (panel A), factor VIIa bound to tissue factor (TF) activates both factor IX and factor X. Activated factor X then activates factor V on the tissue factor bearing cell, complexes with the activated factor V, and converts a small amount of prothrombin (II) to thrombin (IIa). In the priming phase (panel B), the small amount of initial thrombin: activates platelets causing release of alpha granule contents including factor V, activates factor V, activates factor XI, and activates factor VIII by cleaving it from von Willebrand factor (vWF). Cofactors bind to the platelet surface before their respective enzymes. The factor VIIa/tissue factor complex is shut down through the action of tissue factor pathway inhibitor (TFPI) in complex with factor Xa. In the propagation phase (panel C), activated factor IX generated by factor VIIa/tissue factor binds to the activated platelets and subsequently activates factor X. This factor IXa is supplemented by factor IXa generated on the platelet surface by factor XIa. Factor Xa then moves directly into a protected complex with factor Va resulting in a burst of thrombin generation.

Table 1.

Components of the experimental model system.

cells	monocytes	<1 pM	15/μL
	platelets		100,000/μL
procoagulant	prothrombin	1400 nM	100 μg/mL
proteins	factor V	20 nM	7 μg/mL
	factor VII	10 nM	0.5 μg/mL
	factor VIII*	0.3 nM	0.1 μg/mL
	factor IX	70 nM	4 μg/mL
	factor X	135 nM	8 μg/mL
	factor XI	25 nM	5 μg/mL
inhibitors	antithrombin	3000 nM	180 μg/mL
	tissue factor pathway inhibitor	3 nM	0.1 μg/mL
	C1-esterase inhibitor	230 nM	240 μg/mL

* factor VIII is bound to von Willebrand factor.

teract to form a hemostatic plug. Using this technique, we made several important discoveries that have led to a revision of the classic waterfall or cascade hypothesis of blood coagulation as shown in Fig. 2a–c. [21]

The cell-based model of coagulation permitted us to monitor platelet activation (by measuring expression of P-selectin (CD 62)), activation of the co-factors V and VIII, and activation of the zymogens (including prothrombin and factors IX, X, and XI), finally leading to thrombin generation. The components of the cell-based assay are shown in Table 1, which lists the cell types, the procoagulants, and the physiological inhibitors. They were placed in microtiter wells, and the reactions started with the addition of calcium and activated factor VII. Using this assay we discovered the following. First, using cells, as a source of tissue factor and platelets instead of a phospholipid solution, we found that while platelets supplied phosphatidyl serine (PS) that was necessary for coagulation, PS per se could not substitute for all of the platelet contributions to hemostasis. Secondly, platelet activation was dependent upon an intact tissue factor/VIIa pathway that leads to generation of small amounts of thrombin, which is necessary for the activation of factors V and VIII and the activation of platelets. We were also able to confirm that the tissue factor/factor VIIa complex activated both factor X and factor IX, but that the resulting activated factor X and factor IX played very distinct roles in subsequent clotting reactions. The factor Xa remains in the vicinity of the tissue factor-bearing cell and is responsible for platelet activation by generating a limited amount of thrombin on the tissue factor-bearing cell. Factor IXa, on the other hand, is active in converting factor X to factor Xa on the surface of the activated platelet and is responsible for the burst of thrombin generation on the platelet surface. See Fig. 2a–c. This amount of thrombin formed on the platelet surface is sufficient not only to clot fibrinogen but also to activate the thrombin-activatible fibrinolytic inhibitor. [22]

Another finding from our experiments was that factor XI could be activated by thrombin on the surface of platelets without requiring exogenous agents such as dextran sulphate. [23] Thus, the physiological activation of factor XI occurs on the surface of platelets, and thrombin is the physiological activator of zymogen factor XI. The studies clearly showed that the main function of factor XIa is to boost factor IXa generation, which ultimately leads to increased thrombin generation. The role of factor XI in coagulation had always been mysterious since its absence leads to only a mild bleeding tendency (except in particular circumstances). It was previously known that some people require factor XI for normal thrombin generation while other subjects apparently do not require factor XI at all. This observation came from observing that some patients with severe factor XI deficiency had no bleeding tendency even after trauma. These experiments explain why factor XI deficiency is always mild since even without factor XI, the tenase and prothombinase complexes are always present (see Figure 2). Thus, the function of factor XIa is simply to boost thrombin generation when needed. In Figure 2, one can deduce the distinct roles of factors IX and X activated by the tissue factor/VIIa pathway, as well as the role of factor XI in boosting thrombin generation in those people who need it.

Using the cell-based model of coagulation we were also able to work out the mechanism of action of factor VIIa, which is now available commercially and used to bypass inhibitors of factors VIII and IX in hemophilia A and B patients respectively. It is also used to control bleeding in otherwise normal subjects who have sustained severe trauma or surgery or who suffer from a wide variety of acquired conditions associated with excessive bleeding that is not controlled by conventional therapies. These experiments demonstrated that when factor VIIa was given in huge doses, it binds to the surface of activated platelets, which are localized to the site of injury. [24] Factor VIIa on the surface of activated platelets can then *directly* activate factor X to Xa in the absence of tissue factor and leads to an increase in thrombin generation. Factor VIIa not bound to platelets is not active, and this accounts for the safety of factor VIIa, that is, it is active at the site of injury where activated platelets accumulate but not active alone in the circulation. This mechanism also explains the effectiveness and safety of factor VIIa in non-hemophilic patients who are bleeding such as those exposed to serious trauma or surgery and a host of other conditions. This is illustrated by the cartoon shown in Figure 3.

Over the last half-century it has been my pleasure to have witnessed an explosion of knowledge related to blood coagulation and the related clinical conditions of hemorrhage and thrombosis. It has been a rewarding experience to have contributed in a small way to some of this

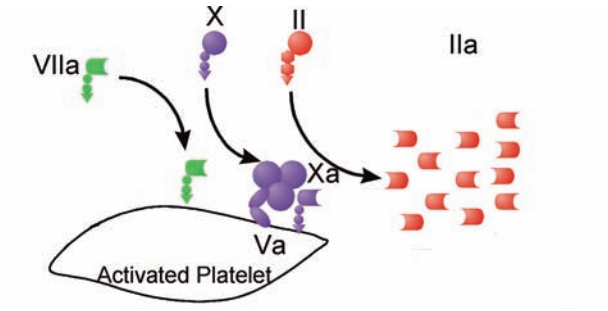

Figure 3. *High dose factor VIIa partially restores platelet thrombin generation*. In hemophilia, deficiency of either factor VIII (hemophilia A) or factor IX (hemophilia B) prevents formation of the factor IXa/VIIIa complex on platelets. Factor VIIa binds weakly to platelets and, even in the absence of tissue factor, can activate factor X on the platelet surface (compare to Figure 2, panel C). This factor Xa can then complex with factor Va to give a burst of thrombin generation.

knowledge. As I write this paper, I notice that I have on my desk a syringe which at one time contained the viral vector encoding the factor IX cDNA that I injected into a hemophilia B patient in an attempt at gene therapy. This study was actually being performed by one of my former fellows, Dr. GC White, II, who gave me the privilege of participating in this event. Although gene therapy is not yet a reality, research in the area is still on-going and will likely be ultimately fruitful. This patient who received the factor IX cDNA experienced a transient increase of factor IX levels for a few weeks without side effects. The patient was not cured of his hemophilic condition, but hemostasis seemed to be improved for a least a few weeks. The empty syringe that still sits on my desk remains a symbol of hope for the future cure of a very devastating disease and is also a symbol of what experimental medicine may hold for the future of mankind.

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