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HISTORICAL SKETCH

Deciphering the mystery of combined factor V and factor VIII deficiency

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The mystery

In 1954 Oeri *et al.* [1] described two young siblings with a lifelong history of a bleeding tendency related to a deficiency of both factor (F) V and FVIII. Reports of additional families in the late 1950s and 1960s [2–6] suggested that the common occurrence of FV and FVIII deficiencies was not a mere coincidence of parahemophilia and hemophilia A. This was further supported by the observation that in five of the eight families reported up to 1969, the affected individuals were descendents of parental consanguinity [5,6].

How could one explain the mechanism of an inherited disorder involving a gene located on the X-chromosome, and another gene located on an autosome? This enigma has attracted the interest of investigators in subsequent years who felt that understanding the pathogenesis of the disorder might shed light on the molecular genetics, synthesis, secretion, and biochemistry of FV and FVIII. The authors of this historical review were among those investigators who entered the arena, Uri Seligsohn in 1967, and David Ginsburg in 1995.

The common precursor hypothesis

In 1967, Uri was training in Hematology at the Sheba Medical Center in Israel and became interested in blood coagulation. Uri was fortunate to have a distinguished mentor, Dr Harry Leiba. Before he left for another medical center, Leiba showed Uri the laboratory data of a woman who bled excessively following delivery and tooth extractions. The patient was found to have an abnormality in the thromboplastin generation test, which was corrected by normal absorbed plasma, a source of FV and FVIII. In addition she had a prolonged prothrombin time and a low FV level. With the assistance of a dedicated laboratory technician, Mrs Eugenia Goldberg, Uri made the diagnosis of combined FV and FVIII deficiency and performed a family study, which revealed that three of the patient's sisters were also affected [6]. It was then that Uri read the landmark paper by Oeri *et al.* [1] and became aware of the common precursor hypothesis (Fig. 1). These authors noted the great similarity between FV and FVIII with both losing their activity in serum, both passing through a Seitz filter and not being absorbed by $BaSO_4$ or $Al(OH)_3$. They postulated that combined FV and FVIII deficiency stems from a defect in the synthesis of a precursor for these proteins. Isolated FV deficiency (parahemophilia) was related to a defect in the conversion of the precursor to FV, and hemophilia A was related to defective conversion of the precursor to FVIII. The precursor hypothesis was later tested by infusing plasma of hemophilia A patients, supposedly containing the precursor, into patients with the combined FV and FVIII deficiency. Three such studies showed no significant increase in FVIII levels [5–7], and hence, the common precursor hypothesis was discarded.

Inheritance

Three family studies suggested that the combined FV and FVIII deficiency is autosomal recessive [5,6,8]. However, some heterozygotes had a bleeding tendency while others did not,

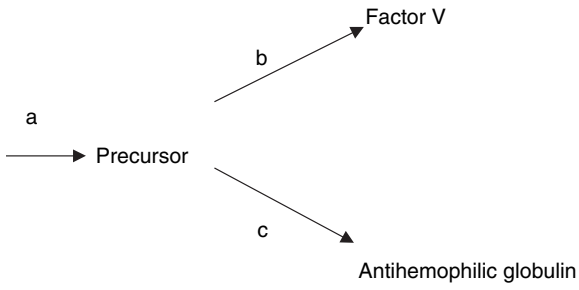

Fig. 1. The common precursor hypothesis for combined factors (F)V and FVIII deficiency (a), FV deficiency (b) and hemophilia A (c). Based on data from Oeri J, *et al.* [Congenital factor V deficiency (parahemophilia) with true hemophilia in two brothers]. *Bibl Paediatr* 1954; **58**: 575–8.

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and some had normal or decreased levels of FV and FVIII. To address the uncertainty regarding the mode of inheritance, Uri's group studied seven families in the early 1980s [9]. Sibship analysis yielded a corrected segregation ratio of 0.24, which was the exact value expected for an autosomal recessive disorder. Moreover, mean FV and FVIII levels in offspring of severely affected patients were significantly lower than normal. Combined FV and FVIII deficiency was thus defined as an autosomal disorder, which is manifested in homozygotes by a severe bleeding tendency highlighted by substantially reduced FV and FVIII levels, and in heterozygotes by partially deficient or normal factor levels.

Deficiency or dysfunction of FV and FVIII

In 1971, Zimmerman *et al.* [10] made the first clear distinction between von Willebrand factor (VWF) and FVIII by using specific antibodies. These authors and the Seligsohn group [9] both showed that VWF levels were normal in patients with the combined FV and FVIII deficiency, analogous to what was found in hemophilia A patients. Whether FV and FVIII antigen levels were reduced, like the factors' activity, was unknown in the 1970s. Uri attempted to address this question by contacting John Giddings and Arthur Bloom in Cardiff, who in the mid-1970s had developed neutralizing antibodies against FV and FVIII. John and Arthur were delighted to collaborate and the results of their examinations suggested that in patients with the combined deficiency, there were measurable amounts of FV antigen, but the results of FVIII antigen measurements were inconsistent [11]. In the 1980s, Uri re-embarked with Ariella Zivelin, his close associate, on an extensive study of FVIII antigen in patients and obligatory carriers. Using an antibody isolated from a hemophilia A patient with a very high titer of a FVIII inhibitor (a gift from Ted Tuddenham) Seligsohn and Ariella found that there was an excellent correlation between FVIII activity and antigen [12]. In those days Paul Tracy and Kenneth Mann had devised a radioimmunoassay for FV and found concordance between FV activity and antigenicity in one patient with the combined deficiency [13]. Together, these measurements were extended to 13 patients and 22 obligatory carriers, showing similar concordance between activities and antigenicities [14]. These data indicated that there was a true FV and FVIII deficiency in patients afflicted by this disorder.

Deficiency of protein C inhibitor

A dramatic development occurred in 1980 when Marlar and Griffin discovered that four unrelated patients with the combined deficiency had no protein C inhibitor in their blood [15]. As activated protein C degrades FV and FVIII, an inherited deficiency of its inhibitor could have been the solution of a 26-year-old enigma. When Uri read this paper, he was somewhat puzzled because in his, and others', experience the decay of FVIII and FV following infusion of cryoprecipitate or plasma was normal and therefore accelerated catabolism of these factors seemed unlikely. Indeed, studies of other patients failed to demonstrate protein C inhibitor deficiency [16,17] and the original findings were finally attributed to the instability of protein C inhibitor following repeated freezing and thawing of plasma [18]. Thus, this beautiful hypothesis turned out to be wrong.

Defective tyrosine sulfation of FV and FVIII as a cause

In 1992, Debra Pittman and Randy Kaufman, at that time at the Genetic Institute in Cambridge, Massachusetts, showed that tyrosine sulfation was required for expression of full FVIII activity [19]. They speculated that the combined FV and FVIII deficiency might result from abnormal tyrosine sulfation. Randy wrote to Uri asking for samples. Once more Uri thought that at last there was a solution to the conundrum and immediately sent the material. The results, however, were disappointing; no defective tyrosine sulfation of FV or FVIII was detected in the plasma [20].

Search of candidate loci for the combined deficiency gene

In 1994, Ariella Zivelin and Uri carried out linkage analysis of genes coding for proteins potentially affecting the catabolism of FV and FVIII, that is, protein C, its cofactors – protein S and FV, and prothrombin. They analyzed intragenic polymorphisms of these genes, anticipating exclusion of their role when two homozygotes from the same family would have different polymorphic alleles, or when offspring of consanguineous parents were heterozygous for the polymorphic alleles. The results indicated that there was no linkage between the disorders and any one of these genes [21].

The beginning of a great collaboration

At about this time (1994), Uri proposed the idea of taking a whole genome/positional cloning approach toward the identification of the elusive gene responsible for the combined FV and FVIII deficiency. Through Randy, Uri began to explore this approach with David, a close colleague of Randy's at the University of Michigan. Randy remained a key collaborator for this work, particularly for the later biochemical and cell biologic characterization (described below). Uri and David realized the potential applicability of the powerful homozygosity mapping [22] to the unique set of patient samples that had accumulated over the years.

Though David was also fascinated by the mystery of this curious disease, the magnitude of a positional cloning approach – relatively new technology in those days – left him cautious. However, Uri's enthusiasm was infectious, and when the two first met face to face and discussed the project, during the ISTH Congress in Jerusalem in 1995, David was convinced. Thus, a wonderful collaboration was launched, which became a turning point in the history of the combined FV and FVIII deficiency. Immediately after the Congress, Uri and Ariella crisscrossed Israel to obtain blood samples from patients and all available

family members, extracted DNA, and shipped the samples to David's laboratory, along with immortalized cells from the patients.

Localization of the gene to chromosome 18q

When Uri first approached David in 1994, the Ginsburg group had just begun to establish the tools for undertaking large-scale genotyping in their laboratory. This effort was led by a very talented postdoctoral fellow, Bill Nichols. Bill had just taken on a project to attempt to map minor histcompatibility genes potentially contributing to graft-vs.-host disease following bone marrow transplantation [23,24]. Having established a system for high throughput genotyping, Bill was in a perfect position to take on another mapping effort, such as identifying the combined FV and FVIII deficiency gene. Indeed several other projects that Bill initiated around that time also ultimately came to fruition, including the positional cloning of the gene responsible for spheroid body myopathy [25], and the gene for primary pulmonary hypertension [26], the latter subsequently becoming a major focus for Bill's independent research program when he joined the faculty at the Cincinnati Children's Hospital Medical Center as an Assistant Professor in 1998.

The combined FV and FVIII deficiency families that Uri had collected turned out to be the key resource that enabled the rapid identification of the responsible gene. Taking advantage of the shortcut of homozygosity mapping, Bill was able to complete the genome scan in all the homozygous affected individuals in only 6 days from the time that the DNA samples arrived from Israel, in the fall of 1995. Scanning a total of 241 highly polymorphic short tandem repeat markers spanning the human genome, Bill found that 13 of 14 affected patients were homozygous for two closely linked markers on the long arm of human chromosome 18 [27]. Analysis of 40 individuals from eight unrelated families ultimately yielded a logarithm of odds (LOD) score of 13.2, with haplotype analysis limiting the candidate interval to a 2.5 cM segment. Bill presented these exciting results at the 1996 American Society of Hematology meeting and this initial mapping study was published in February 1997 [27]. However, there was serious competition. The very capable group of Ted Tuddenham, Stylianos Antonorakis and Marguerite Neerman-Arbez had also collected a sizable group of families with the combined deficiency and were hot on the trail. Their paper confirming our findings in 19 families from Iran, Pakistan and Algeria was published in July 1997 [28].

The location of the gene on chromosome 18q excluded all known hemostatic proteins, and suggested that the gene defect affects a process common to the biosynthesis of both FV and FVIII. Already at this stage, it was realized that there was linkage disequilibrium between the disease locus and two independent founder haplotypes, one for all five affected Tunisian Jewish families, and another for all four Middle Eastern Jewish families.

The discovery of the *LMAN1* (ERGIC-53) gene and the first two mutations

Positional cloning and analysis of genes within the candidate genetic interval identified the disease gene as that encoding the 53 kDa protein known at the time as ERGIC-53 (endoplasmic reticulum Golgi intermediate compartment). Though ERGIC-53 was commonly used by cell biologists as a marker for the intermediate compartment between the ER and Golgi, its function was entirely unknown [29]. Immunofluorescence studies and Western blotting of immortalized cells from the patients revealed complete absence of ERGIC-53 antigen. DNA sequence analysis identified two homozygous mutations (corresponding to the two previously identified haplotypes) accounting for all of the studied patients. One was a G insertion predicting a frameshift and resulting in a truncated protein that was common to all Middle Eastern Jewish families. The other mutation was a T to C change at a donor splice site, also predicted to disrupt the protein, that was shared by all Tunisian Jewish patients. Uri's team subsequently found out that all the Tunisian Jewish families originated from the island of Djerba, where a Jewish community had lived for more than 2000 years [30]. A survey of Djerba Jews presently living in Israel for the mutation by restriction analysis showed that the frequency of the mutant allele was 0.011 in this population.

Worldwide search for additional mutations

At that stage in 1998 the team was ready to search for additional mutations in *LMAN1* (the current name of ERGIC 53). Uri and David contacted friends and colleagues in Japan, Italy, France, Turkey, Venezuela, and the USA and asked them to send DNA from their patients. The team's enthusiasm was probably contagious because within a short time the samples were received. At this stage we also joined forces with our former competitors (Neerman-Arbez *et al.*), which yielded another rich source of patient material. Seven novel *LMAN1* mutations were identified in our patient samples and another 13 in those of Neerman-Arbez *et al.* All mutations were predicted to result in complete absence of the LMAN1 protein. The two papers were published back to back in *Blood* [31,32]. All noted that in approximately 30% of the families no mutation in *LMAN1* was discernable, implying the existence of an additional genetic locus. This hypothesis was also supported by linkage data in two families in whom there was no *LMAN1* mutation [31], and by the finding of normal LMAN1 protein levels in cells derived from a number of *LMAN1* mutation-negative patients [32].

The discovery of the *MCFD2* gene

Bill Nichols left David's laboratory in 1998 to start his own independent laboratory at the Cincinnati Children's Hospital Medical Center, though he remained a key collaborator for subsequent studies. A new postdoctoral fellow, Bin Zhang,

joined David's laboratory in the summer of 1998. Bin focused on the families that we had collected, together with our international team of collaborators, who lacked mutations in the *LMAN1* gene. This work was aided greatly by progress in the human genome project. Homozygosity mapping in 19 patients from 10 families first mapped this new locus to the short arm of human chromosome 2 and subsequently identified the responsible gene as *MCFD2*. Seven mutations, 3 frame shift, 2 splice site and 2 missense were identified in *MCFD2*, accounting for the vast majority of the remaining patients who lacked *LMAN1* mutations [33].

The function of LMAN1 and MCFD2

In vitro studies [29,33–36] indicated that formation of a calcium-dependent complex of LMAN1 and MCFD2 is an essential step for the efficient transport of FV and FVIII from the ER to the Golgi. This complex serves as a specific cargo receptor for the selective packaging of FV and FVIII into COPII-coated vesicles that bud from the ER for transport to the Golgi. To date, no other cargo proteins dependent on this pathway have yet been identified. At present, a mutation in either the *LMAN1* or *MCFD2* gene has been identified in 71 of 76 studied families with combined FV and FVIII deficiency. Among the remaining families in whom no mutation was identified, three were because of misdiagnosis and two likely carry an *LMAN1* or *MCFD2* mutation that was missed during sequencing [37]. Thus, the current data suggest that mutations in *MCFD2* or *LMAN1* account for all cases of combined FV and FVIII deficiency.

Perspective

Combined FV and FVIII deficiency is an instructive example of a hemostatic disorder in which the genes coding for the deficient proteins are normal, with the disease instead being caused by defects in post-translational processing. Similarly, mutations in the genes encoding gamma-carboxylase or the vitamin K epoxide reductase have been shown to cause deficiency of all vitamin K-dependent clotting proteins [38]. Both of these disorders are also wonderful examples of how the study of rare human diseases can provide fundamental insight into basic biologic processes. Indeed, the LMAN1/MCFD2 complex is the first example of a specific cargo receptor for the transport of secreted proteins from the ER to the Golgi. Finally, in addition to the basic cellular mechanisms uncovered by these studies, identification of the *LMAN1* and *MCFD2* genes may also provide a target for the development of novel anticoagulant therapies in the future, as well as potential tools for more efficient production of recombinant FVIII and FV.

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