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The biology of von Willebrand factor and factor VIII-regulated release

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The clinical association of factor VIII (FVIII) deficiency with both hemophilia A and von Willebrand disease (VWF) has been recognized for more than 50 years.[1-3] However, the mechanism by which regulated secretion of both proteins occurs, remains controversial.[1] Since FVIII is deficient in both hemophilia A and severe von Willebrand disease (VWD), it was not surprising to find that the reason for this observation was that VWF (VWF) served as a carrier protein for FVIII.[1] In the absence of VWF, the unbound FVIII is rapidly proteolyzed — a process that reduces its plasma half life from 12 hours to one to two hours. Since patients with severe VWD still had a residual level of FVIII between 3 and 8 IU/dL in the absence of detectable VWF, infusion of VWF resulted in a delayed rise in plasma of FVIII over the subsequent 12 to 24 hours, this was assumed initially to be stimulation of FVIII synthesis, although today, it is recognized that FVIII synthesis is probably progressing normally and it is only in the presence of transfused VWF that the FVIII survival can now normalize and give the clinical picture of the delayed rise in FVIII. The purpose of this review is to establish the basis for how a regulated storage pool, of both VWF and FVIII, is established.

The release of VWF and FVIII with DDAVP

For more than 20 years, it has been known that DDAVP given intravenously (or subcutaneously or intranasally) will result in an instantaneous increase in both VWF and FVIII.[4,5] This occurs in normal individuals as well as patients with mild or moderate deficiency of VWF or FVIII. In patients with severe VWD, neither FVIII nor VWF are released in response to DDAVP. In severe hemophilia A, VWF is released, but there is no release of FVIII. Figure 1 demonstrates the release of VWF and FVIII in patients with mild VWD and mild hemophilia A (panel A and B).

Delayed rise in plasma FVIII following VWF infusion

A number of years ago, a method was established to purify plasma VWF for therapeutic purposes in France.6 Unlike other concentrates containing VWF, this concentrate contains little or no FVIII. As shown in Figure 1 Panel C, when this concentrate is infused into a patient with severe Type 3 VWD, the VWF increased immediately, but the FVIII slowly elevates over the next 12 to 24 hours. The cause for this rise is the change in plasma half-life of FVIII in the absence of VWF to its normal half-life in the presence of VWF. The kinetics of this response has limited clinical utility of this concentrate somewhat because in the acute bleeding situation, the delayed rise in FVIII is sometimes inefficient for immediate normalization of hemostasis.

Cellular synthesis and storage of VWF

The cellular synthesis of VWF is illustrated in Figure 2. The transcript for VWF results in the synthesis of pre-pro-VWF, that is directed to the endoplasmic reticulum by a 22 amino acid signal peptide. Pro-VWF is synthesized as a C-terminal dimer that is partially glycosylated and then transferred to the Golgi where it undergoes final glycosylation and carbohydrate trimming, N-terminal multimerization, and furin-mediated cleavage of the VWF propeptide, VWFpp. Both the VWFpp and VWF are packaged into secretory granules in endothelial cells (Weibel-Palade bodies) and megakaryocytes/platelets (α-granules). This process proceeds in a similar manner regardless of whether mature VWF and the VWFpp are produced in *cis* (single DNA construct) or *trans* (as to DNA constructs).[7] While it has been recognized for many years that the VWFpp is required for multimerization, it was not clear if it was the multimerization process, itself, that caused VWF storage or if storage and multimerization were two independent processes.[8-11] Rosenberg and coworkers demonstrated

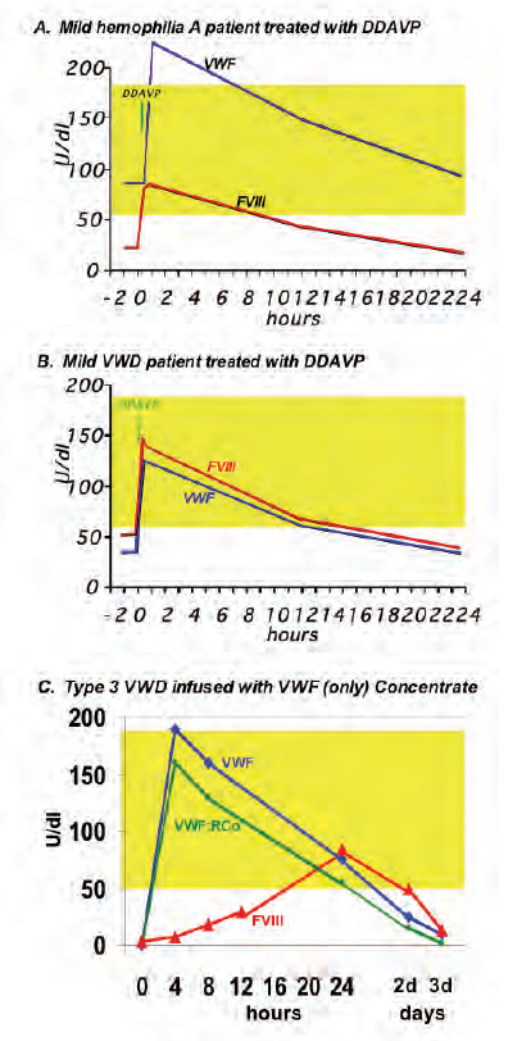

Figure 1. Clinical response to DDAVP in hemophilia A and VWD or VWF replacement in severe VWD. Panel A. A patient with moderate hemophilia A is given a standard dose of DDAVP. There is an immediate and synchronous rise in both VWF and FVIII. Panel B. A patient with moderate VWD is given a similar dose of DDAVP. Again, there is an immediate and synchronous rise in both VWF and FVIII. Panel C. A patient with severe VWD (undetectable VWF and 3-5 IU/dL plasma FVIII is given a VWF concentrate that contains minimal amounts of FVIII. While the VWF:Ag and VWF:RCo increase immediately, the FVIII rises slowly reaching a maximum within the normal range at 24 hours and the survival of VWF and FVIII is then similar over the next 48 hours. This FVIII is endogenously produced in the VWD patient and it is the normalization of VWF that thereby normalizes its disappearance and the apparent delayed rise in FVIII. (used by permission, RR Montgomery)

that a mutation in the D1 region of VWFpp (Y87S) results in a mature VWF protein that is not N-terminal multimerized.[12] Yet, the protein was still stored in secretory granules. Studies by Haberichter and coworkers used portions of the cDNA of canine VWFpp in *cis* or in *trans* with the cDNA for human VWF.[11;13] She was able to demonstrate the converse of the Rosenberg experiments. In these studies, the VWF was fully multimerized but was not packaged into secretory granules. On closer examination of these cells, the VWFpp was, in fact, stored but the human VWF was not.[13] Using site directed mutagenesis, Haberichter and coworkers demonstrated that when the canine VWFpp was mutated at amino acid 416 from the glutamine that is in the canine sequence to the arginine in the human sequence, storage of human VWF was reestablished. In a similar manner, they were able to study human VWF at position 869 and change the threonine that is present in the human sequence to alanine or to the alanine that is present in the canine sequence and again, reestablished human VWF storage. In each case, canine VWFpp was stored while VWF storage was not stored. Furthermore, in all cases, the VWF was multimerized. Thus, the storage process is initiated by the VWFpp and only if there continues to be an association between VWFpp and VWF is there effective storage of VWF regardless of whether the VWF is multimerized or not.

How does FVIII undergo regulated release?

As demonstrated previously in Figure 1, DDAVP releases both VWF and FVIII and the kinetics of this release appears to be identical. How this secretory pool of FVIII is established is still not clear. The message for FVIII has been difficult to examine because of the low levels of FVIII produced. While it has been presumed that FVIII is made in the hepatocyte, message has not been demonstrated in hepatocyte cell lines.[14,15] Clinically, even in the face of profound liver failure and hepatocellular death, FVIII levels are maintained or even increased.[1,16] While liver transplant in hemophilia A has reestablished normal levels of plasma FVIII,[17] the cell within the lever producing this FVIII is not clear. Cultured endothelial cells have not been demonstrated to produce FVIII protein in measurable amounts although two recent studies have suggested that the message may be present *in vivo* in selective endothelial cell populations and, at least in the mouse, sinusoidal endothelial cells.[14,15,18]

Rosenberg and coworkers have demonstrated that if the FVIII gene is introduced into a cell that is producing and storing VWF, FVIII will traffic together with this VWF and be stored in secretory granules.[2,16,19] Mutations in VWF that effect FVIII binding (Type 2N VWD) can be demonstrated to also block the trafficking of FVIII together with VWF when co-expressed *in vitro*.[2] If FVIII is expressed in normal umbilical vein endothelial cells, FVIII is stored in Weibel-Palade bodies. If both VWF and FVIII are expressed in AtT-20 cells, both proteins are stored in the same granules.

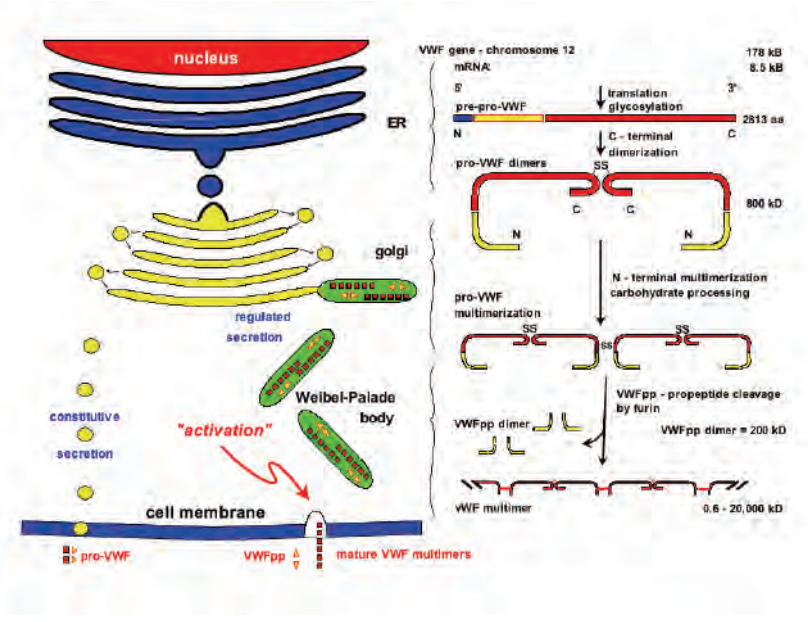

Figure 2. Intracellular Synthesis of VWF. VWF is synthesized in endothelial cells and megakaryocytes. In the endothelial cell, Weibel Palade bodies containing VWF are the secretory stores that release VWF into plasma following DDAVP. The signal peptide directs pre-pro-VWF to the endoplasmic reticulum where processing is initiated as a C-terminal linked dimer. Pro-VWF is then transported to the golgi where multimerization is facilitated by the VWF propeptide, VWFpp. The VWFpp is cleaved from mature VWF by furin, a dibasic peptidase. At acidic pH, the VWFpp and mature VWF multimers are sequestered in secretory granules (e.g. Weibel-Palade bodies). Following regulated release of these granules, VWF multimers and the VWFpp are released into plasma where, at neutral pH, the two proteins disassociate and circulate independently *(used by permission RR Montgomery).*

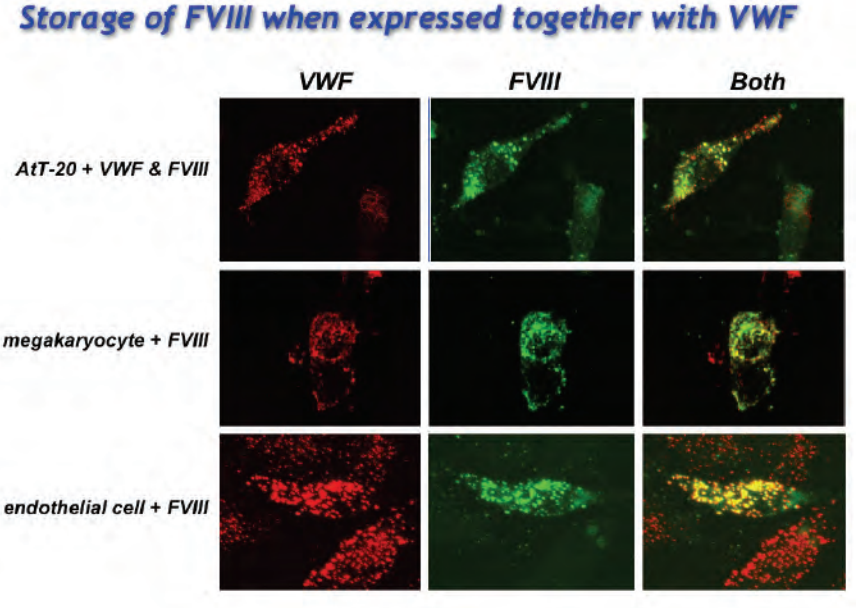

Figure 3. FVIII co-expression with VWF in AtT-20 cells, megakaryocytes, and endothelial cells. The AtT-20 cell can be transfected with the cDNA for VWF and the cDNA for FVIII. The association of FVIII with VWF enables the storage and release of both proteins. Megakaryocytes normally synthesize VWF and store VWF in α-granules; FVIII is not produced normally in megakaryocytes. If FVIII synthesis is induced in megakaryocytes through transduction of FVIII cDNA, FVIII now co-stores in the ·-granule. Endothelial cells normally synthesize, store, and release VWF. In cultured umbilical vein endothelial cells, FVIII is not expressed, only VWF. If FVIII expression is transduced in these cells, FVIII is synthesized and the FVIII and VWF are stored, and are released together following agonist stimulation. These stores would be released by DDAVP and could account for the *in vivo* observed response, but FVIII synthesis by endothelial cells remains controversial *(used by permission, RR Montgomery).*

Similarly, FVIII expressed in megakaryocytes results in storage of FVIII in α granules although recent studies by Poncz and coworkers suggest that some of this storage might still occur in the absence of VWF. Figure 3 demonstrates the co-storage of FVIII in endothelial cells, megakaryocytes, and AtT-20 cells if FVIII is synthesized in the presence of VWF.

Can the regulated secretion of FVIII be reestablished through protein replacement?

Montgomery and Gill studied patients with severe hemophilia A and severe Type 3 VWD in the face of FVIII and VWF replacement respectively. Figure 4 demonstrates the results of these studies. When a hemophilia A patient receives recombinant FVIII replacement on a prophylactic basis, near normal lev-

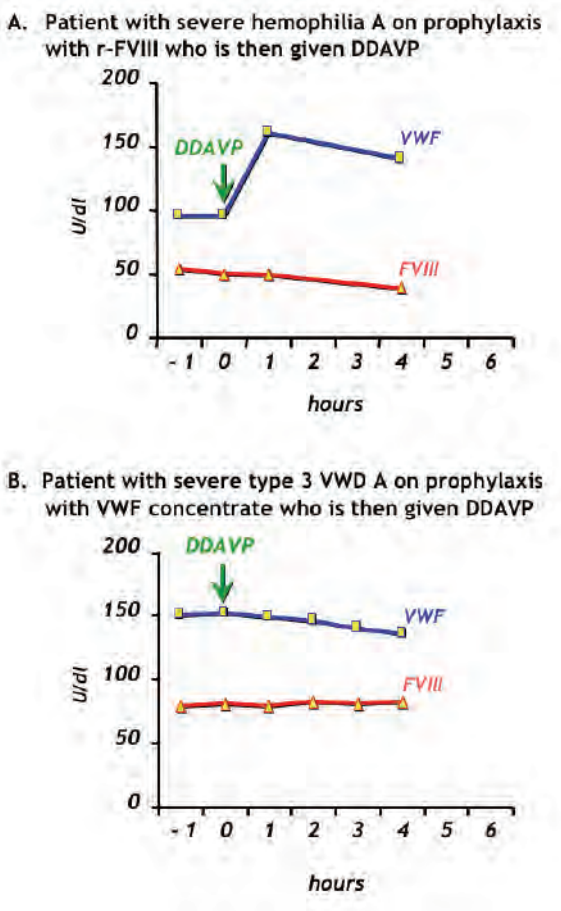

Figure 4. DDAVP administration to patients with severe hemophilia A or VWD following therapeutic replacement. Panel A. A patient with severe hemophilia A is on prophylaxis and the FVIII level is being supported by infusion of recombinant FVIII. When DDAVP is administered to this patient, plasma VWF is increased, but there is no change in the plasma FVIII. This suggests that the DDAVP releasable pool cannot be re-established through uptake from plasma. Panel B. A patient with severe VWD received VWF concentrate (minimal FVIII) daily for three days. The endogenous plasma FVIII level was normalized by the VWF replacement. When DDAVP is administered, neither the plasma VWF nor the plasma FVIII is increased. Thus, the endogenous synthesis of FVIII alone, even in the context of normal plasma VWF, establishes a DDAVP releasable pool of FVIII. DDAVP release of FVIII requires endogenous synthesis of both VWF and FVIII *(used by permission RR Montgomery).*

els of FVIII can be identified in plasma. If he was then given DDAVP, VWF was released but there was no increase in plasma FVIII (Figure 4.A). Thus transfused FVIII does not have access to intracellular stores of VWF in order to form this intracellular association. In the second experiment, a patient with Type 3 VWD was given just replacement therapy with VWF. After several days, not only were VWF levels normal, but also plasma FVIII was normal due to normalized survival of endogenously synthesized FVIII. When this individual was given DDAVP, neither VWF nor FVIII was increased in plasma Figure 4.B). Thus the reestablishment of normal FVIII in a Type 3 VWD patient does not restore a DDAVP-releasable pool of FVIII. Taken together, these studies demonstrate that a DDAVP-releasable pool of FVIII is only established when both VWF and FVIII are synthesized endogenously.

Figure 5 provides three scenarios by which a DDAVP releasable pool of FVIII could be established. One of those could be ruled out on clinical grounds since the time course of FVIII release is too quick for there to be normalization of survival. Normalization of survival is what occurs in the Type 3 patient who was given VWF. This takes 8 to 12 hours and could not account for the immediate rise in FVIII (Figure 5.A). A second model would be FVIII and VWF being synthesized in adjacent cells and for there to be cellular transfer of the FVIII into the VWF storage compartment of the second cell (Figure 5.B). This will be discussed in more detail in the next paragraph. The third alternative is that FVIII and VWF are both synthesized in some endothelial cells (not necessarily all endothelial cells) and that it is this coordinate synthesis within a single cell population that establishes the regulated secretion of FVIII (Figure 5.C). This does not necessarily mean that all of plasma FVIII must come from endothelial cells, but only the stored FVIII that is released by agonists such as DDAVP.

What if VWF and FVIII were made in adjacent cells?

We undertook studies in which a cell synthesizing and storing VWF (endothelial cell) was co-cultured with a cell (AtT-20 cell) that was stably expressing FVIII. Figure 6 demonstrates the results of these studies. While FVIII could be demonstrated immunochemically within these AtT-20 cells (green) and VWF could be demonstrated to be made and stored in endothelial cells (red), none of the endothelial cells were capable of taking up this FVIII from the culture media or from cell-cell contact. The limitation of this study is that we might not be using the physiologic cells for co-culture, but at least do not demonstrate transfer from one cell to the next.

Implications concerning VWF and FVIII cellular synthesis. Studies on the biology of VWF and FVIII synthesis is important, but there may be even greater implications that are related to the future gene therapy for hemophilia A or Type 3 VWD. There have been at least 3 gene therapy trials to treat hemophilia A.[20–23] Perhaps in hemophilia, FVIII needs to be expressed in a VWF synthesizing cell and secondly, for VWD that VWF needs to be produced in a FVIII synthesizing cell. In studies by Poncz and coworkers,[24,25] Shi and cowork-

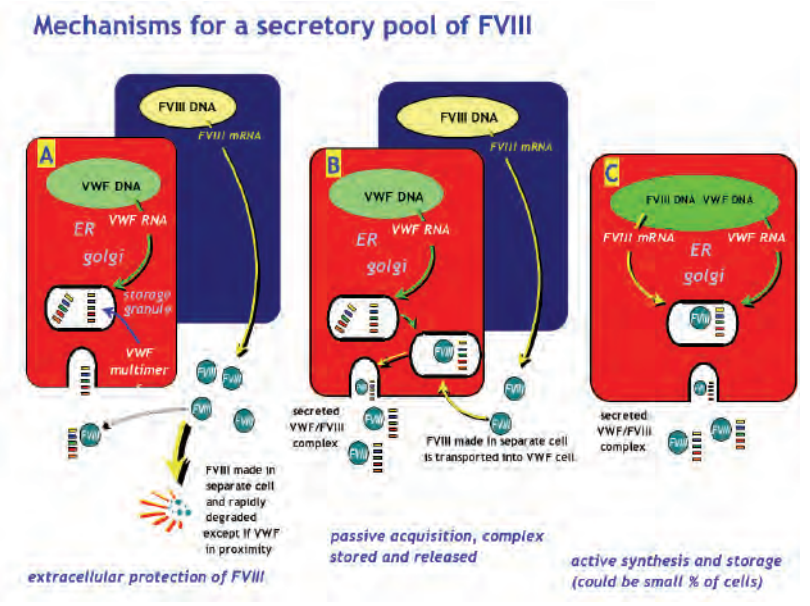

Figure 5. Three models for establishing a DDAVP releasable pool of FVIII. Panel A. This model characterizes that the released VWF would increase the plasma FVIII, but Figure 1.C. illustrates that this increase would take 12-24 hours and would not account for an immediate rise in FVIII. Panel B. This model illustrates what would happen if VWF was stored in one cell and a second cell synthesized FVIII. If the FVIII uptake by the VWF-synthesizing cell was from plasma, this is ruled out by the clinical study in Figure 4.A. If this was uptake from an adjacent cell producing FVIII, this is unlikely given the results to be discussed in Figure 6. Panel C. This panel illustrates what would happen if both proteins were synthesized by the same cell that cell stores VWF; FVIII and VWF would be released together. This would fulfill the clinical observation of co-ordinate release, but it is not yet established if this is the mechanism to explain the physiologic response *(used by permission RR Montgomery).*

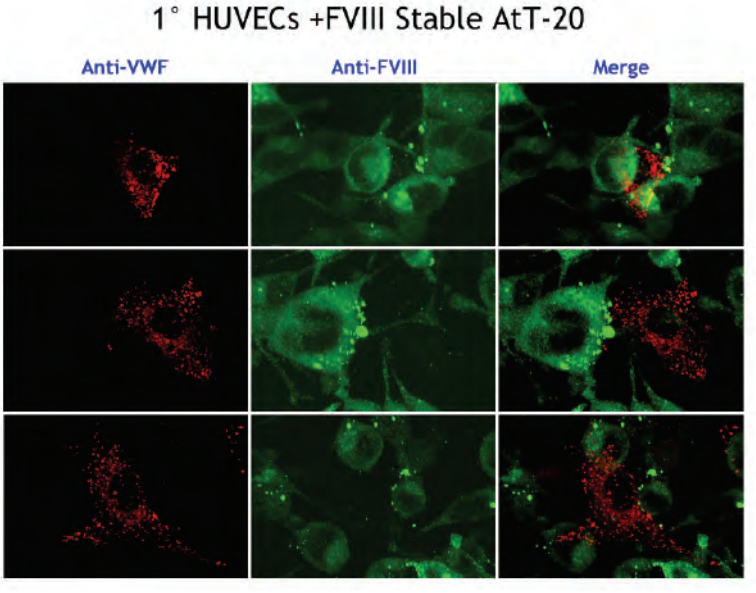

Figure 6. Co-culture of endothelial cells (VWF) with AtT-20 cells (FVIII). Endothelial cells normally synthesize VWF and store the VWF in Weibel-Palade bodies. AtT-20 cells were stably transfected with FVIII cDNA. These cells synthesize and secrete FVIII but no not store FVIII in regulatory, secretory granules. These two sells are co-cultured so that cells synthesizing and stroring VWF are directly adjacent to cells synthesizing and secreting FVIII (AtT-20 cells). Confocal immuno-microscopy demonstrates synthesis and storage of VWF by the endothelial cells and FVIII synthesis by the AtT-20 cells but there is no evidence of FVIII in any of the VWF regulated secretory granules through cell-to-cell transfer or uptake from the media *(used by permission RR Montgomery).*

ers,[26] and Wilcox and coworkers,[27] gene therapy of hemophilia might be possible using megakaryocytic expression of FVIII. Furthermore, the endothelial cell might be a potential target for such gene therapy for hemophilia, although expressing FVIII in a known antigen-presenting cell could be problematic.

Two clinical studies impact on the expression of FVIII with VWF. Ponder and coworkers[28] carried out gene therapy using a hepatocyte-specific promoter and could not demonstrate the establishment of a DDAVP releasable pool even though FVIII levels were re-established. Ragni and coworkers[29] have also studied a patient with hemophilia A who received a liver transplant – after which, the plasma FVIII was normalized. When this patient was given DDAVP, there was no release of FVIII. In the first experiment, directing FVIII expression to the hepatocyte did not enable the establishment of a releasable pool. In the second, even full liver transplantation did not establish a DDAVP releasable pool. Further research and investigation

will be required to understand this process, and the importance of this process to hemostatic regulation.

Conclusions

In this manuscript, we have attempted to summarize some of our current understanding of how the releasable pool of VWF and FVIII are established. A number of variables are still not clear, but on the basis of clinical observations, it appears that this releasable pool requires the endogenous synthesis of both VWF and FVIII.

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