

THE HEMOPHILIA BULLETIN

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San Diego is a wonderful venue for the Society. It's a long trip for Europeans, but they seemed to enjoy the winter sunshine.

At the "Corporate Friday" session called, "**Perspectives on the Control of Hemostasis in Patients with Excessive Bleeding**" sponsored by Novo Nordisk, Maureane Hoffman of Duke University reviewed her idea of the interaction of **coagulation factors on the surface of CELLS**, as follows:

- (1) Tissue-factor-bearing cells, with activated factor VII, activate factor IX and also activate factors V and X. A tiny amount of thrombin is generated.
- (2) The thrombin activates factors XI, VIII and V on the platelet surface.
- (3) On the platelet surface, VIIIa attaches IXa, more X is activated to Xa which, with Va, converts more prothrombin to a large amount of thrombin. Thrombin activates GP1b receptors on platelets which then activate platelet GP IIb-IIIa receptors which attach fibrinogen, which converts to fibrin.

She answers the old question, why do persons with hemophilia A or B bleed, why isn't the alternative pathway of coagulation, through factor VII, enough? Tissue-factor-bearing cells CAN make Xa, but, it can't diffuse *en masse* to platelets, it's cut down by tissue factor pathway inhibitor (TFPI) and anti-thrombin III. Platelet surface activation demands the presence of factors VIII and IX for substantial conversion of prothrombin to thrombin.

Activated factor VII, in high, non-physiologic doses, pushes some generation of thrombin in tissue-factor bearing cells and on platelets.

Dr. Hoffman also reminded us that both **temperature and pH** strongly influence hemostasis. Platelet adhesion is markedly diminished when temperatures fall from 37° C to e.g. 33° C, *in vitro*. Beware of hypothermia in surgical suites and in patients with trauma! The rate at which Xa and Va generate thrombin falls with a small drop in pH, as in acidosis.

Another program, sponsored by the University of North Carolina at Chapel Hill and by Ipsen, was titled, "**Can We Improve on Nature?**" It startled me. I knew that the genome is vastly imperfect, but I had not faced the issue emotionally. With such a slap-dash genome, how have we living creatures managed to survive at all? Have we not evolved efficiently? Maybe we've evolved despite inefficiencies.

My reaction was triggered by Randal Kaufman's report on the **inefficient secretion of factor VIII** out of the cell. The expression of FVIII by recombinant cells is much lower than that of other proteins including closely-related factor V. If a short segment from factor V is substituted for the parallel locus in FVIII, the expression of FVIII is improved about three-fold, to the level at which factor V itself is expressed. Furthermore, making a single mutation in the targeted area (Phe309Ser) improved expression as much (three-fold) as splicing in the entire segment. One crummy nucleotide! So why doesn't Mother Nature do it that way? Does she know something we don't know, that it wouldn't be good for us, or, is she just haphazard? (Let's hear it for the sloppy approach to mothering!) Or is there much more disorder in life than "natural selection" suggests?

The expression of B-domain-deleted FVIII within transfected cells (for recombinant concentrate, for gene therapy) is high but secretion is reduced, it's retained inside the cell even more than full-length FVIII. One can add back a certain bit of the B domain and improve secretion of BDD-deleted FVIII more than ten-fold.

FVIII can be engineered to survive longer in the circulation. Steven Pipe described creating two mutations to engineer a disulfide bridge

between the A2 and A3 domains of FVIII, thus preventing cleavage of the A2 subunit after thrombin activation. The new molecule retained 90% of its activity after thrombin activation. The mutant super-molecule would be beneficial for gene therapy for hemophilia, where only a small amount of FVIII might be expressed and prolonged survival would be useful.

The **activity of factor IX can be improved** several ways, as Darrel Stafford explained. A more-active molecule would be especially desirable in gene therapy, where only a low level of expression is achieved. In one of his designer-molecules, a segment (the first EGF-domain) is replaced with that of factor VII, resulting in tighter binding to FVIII. In dogs, this molecule retains activity much longer than wild-type factor IX. Carboxylation of the molecule can be maximized by substituting the propeptide of prothrombin. A few other mutations, each created to raise activity, have been combined in a single molecule for maximum activity.

The big worry in all this, of course, is that a deliberate mutation might increase the immunogenicity of a molecule. Labs can test *in vitro* for increased activity and *in vivo* in dogs for increased survival. Tests for increased immunogenicity just don't exist.

Attempts also are being made to **decrease the immunogenicity of FVIII**, the ragweed of clotting factors, by identifying the epitopes to which inhibitor antibodies commonly attach and by making substitutions, for example, from porcine FVIII.

A scientific subcommittee meeting was devoted to the biology of **von Willebrand factor**. Bob Montgomery reviewed the **functions of the VWF propeptide**. It's necessary for multimerization and for storage of VWF in Weibel-Palade bodies in endothelial cells and in alpha granules in platelets. The normal propeptide will go into storage alone but the mature subunit will not go into storage without the propeptide. Mutant propeptides may prevent storage. Multimerization is not necessary for storage. DDAVP releases the stored propeptide-VWF-FVIII complex, then the propeptide is cleaved.

It's clear that VWF is made in megakaryocytes and endothelial cells but it's still unclear where FVIII is made. (According to Hollestelle et alia, it's in hepatic sinusoidal endothelial cells, Kupffer cells, renal glomeruli and tubular epithelial cells – *Thromb Haemost* 2001; 86:855-61.) If FVIII and VWF are expressed in the same recombinant cell, both go into storage. If FVIII and VWF are expressed in different cells in the same culture medium, FVIII is not stored. Dr. Montgomery suggests that co-expression of FVIII and VWF ought to be considered for gene therapy. (It makes sense to think that we make FVIII in the same cells as VWF. I'm betting on endothelial cells. I think of FVIII, delicate in so many ways, as a fan-dancer, teasing us.)

Ian Peake reviewed ongoing studies of **type 1 VWD**. There's been a little progress since the ISTH meeting of July 2003. **Mutation analysis** is complete in 132 of the 152 index cases in the European study; 95 patients have mutations, including six patients with definite type 2 mutations, and some patients with two or three mutations. Dr. Peake is beginning to characterize the effects of mutations in specific regions when there are multiple examples. An instance is the D4-CK region, with 17 mutations, characterized by definitely reduced levels of VWF (mean 23% VWF:RCO, 28% VWF:Ag) and slight abnormalities of multimers found with the sensitive method of Ulrich Budde. As reported in July, a mutation is more likely to be found if VWF levels are quite low than if they are in the low-normal range. More than 90% of index cases with VWF:RCO levels below 10% have mutations whereas only half of index cases with VWF:RCO over 50% have mutations. It's a trend, not an absolute. It's unsettling to me to know that so many patients with VWF levels within the normal range do, indeed, have mutations of the VWF gene. No wonder diagnosis is so difficult!

Using gene markers, **linkage of the phenotype of VWD to one VWF gene** can be shown in 70% of affected families. On average, lower VWF:Ag levels are found in affected persons in linked families than in affected persons in unlinked families. Mutations were found in 23 index patients from unlinked families, suggesting that mutations arose *de novo*. One frequent mutation has been shown to be penetrant in 70% of persons but non-penetrant in 30%. Dr. Peake hopes to look for mutations in phenotypically-unaffected members of families, perhaps even controls.

The findings of the EU and the Canadian studies on type 1 VWD were further reported in oral sessions.

Anne Goodeve reminded us that **ABO blood group** accounts for 30% of the variance in VWF levels. In the EU study, 67% of the index cases have group O blood (vs 38% in the EU population.) Among index cases found to have a mutation, 61% had blood group O and 39% were non-O. Among those NOT found to have a mutation, 76% were group O and 24% non-O. Linkage was confirmed for 56% of blood group O index cases and for 78% of non-O index cases.

The **Canadian study of type 1 VWD** has found that group O blood predominates in index patients with near-normal VWF levels but is proportional to the general population in patients with definitely low VWF levels. Linkage studies show co-segregation of VWF gene and phenotype in 30 families, definite non-linkage in 6 families and inconclusive results in 78 families. (Definitive linkage studies require larger families than were studied.)

In other oral presentations, Paula James of Canada described the **suppression of nonsense mutations in hemophilia A with an aminoglycoside**, gentamycin. Aminoglycosides are known to allow some reading of DNA past a stop-codon (nonsense mutation) by inserting a random amino acid. Three patients with severe hemophilia A and two with severe hemophilia B, and nonsense mutations, were treated with intravenous gentamycin (off-label) in typical therapeutic doses for three days. Using very sensitive assays, they were able to detect a transient rise of FVIII from 0.6% to 1.4% on the third day in one patient with hemophilia A, and a rise of factor IX from 0.9% to 2% in one patient with hemophilia B. The two patients who responded had mutations in non-conserved parts of the relevant genes. The three non-responders had mutations in highly-conserved regions. Although these elevations of factor levels were minimal, the result was encouraging. Gentamycin itself is not a candidate for prolonged use because of its high level of toxicity. An agent with similar action but less toxicity may now be sought. With luck, one might find something to take orally.

Most human **gene therapy** trials for hemophilia have paused for reconsideration, with the expectation of eventual, if not rapid, success. Meanwhile, **new approaches** are being evaluated. An animal experiment was described by Ling-fei Xu and colleagues (St. Louis, Philadelphia, Chapel Hill). He injected a retroviral vector with a canine B-domain-deleted FVIII gene into newborn hemophilia A mice. The mice produced FVIII at normal or high plasma levels for months and did not develop inhibitors to the canine protein or vector. He also treated two newborn hemophilia A dogs with this construct. The dogs had sustained high plasma levels of FVIII for three months as of early December. Exposure to the gene therapy construct when an animal's immune system is immature may lead to tolerance. Mice, however, have much less mature immune systems at birth than do humans. This approach may have practical application eventually with prenatal diagnosis in humans.

Two approaches implied that the bone marrow might be a useful target organ. David Wilcox and colleagues (Milwaukee, Chapel Hill, France) used a retroviral vector with B-domain-deleted FVIII to transduce mouse bone marrow cells, obtaining **transgenic megakaryocytes**. Immunofluorescent staining demonstrated that FVIII was present in the same distribution as VWF. Electron microscopy showed FVIII and VWF in alpha granules. Platelets derived from the megakaryocytes also contained FVIII and VWF in alpha granules.

Helen Yarovoi (of the same group) extended the idea. She produced transgenic hemophilia A mice who expressed FVIII in the platelets (but not in the plasma). On challenge, e.g. tail vein clipping, hemostasis usually was good. FVIII may do well in platelets. It may have a longer half-life than in plasma. It may be protected from circulating inhibitors and may be delivered in a concentrated dose to the site of injuries.

Fiona Rowle of Canada fed hemophilia A mice FVIII (human or canine) orally before infusing them, or attempting gene therapy, with the corresponding FVIII. **Oral feeding induces partial tolerance**. The idea has been reported before but bears repeating. Animal studies do not always translate to humans, but, one wonders whether it would be beneficial to inject FVIII into the amniotic fluid when a fetus is diagnosed as having hemophilia A, especially with a genotype with a high risk of inhibitor formation. The fetus swallows some amniotic fluid.

Genotypes have been confirmed so far in 94 of 172 patients with hemophilia A and inhibitors in the US multi-center **inhibitor** study, as reported by Margaret Ragni. Her most startling observation was that some patients with **large deletions** were easily made tolerant. Arthur Thompson of Seattle commented that all eleven patients known to him who had large deletions, inhibitors and attempts at tolerance had failed. It's an important issue. Should tolerance be attempted on patients with large deletions? Many people say yes, and Dr Ragni's early report confirms that opinion. On the other hand, if we knew that a patient had an unfavorable genotype, we might approach tolerance differently. We might start it with an intensive protocol, such as that Bonn or Malmo, or, if we'd treated a patient for a few months with no response whatsoever, perhaps we'd quit and spare him more discomfort and expense. At one session, I heard a familiar question from the audience, a doctor asked, given that his patient had been on tolerance for a year without response, when should he give up? My only large-deletion patient was completely unresponsive to our attempt at tolerance. His non-response was evident early, at three months, when his inhibitor had not budged, nor did it fall after nearly a year's treatment. I beg colleagues in Germany and Swede, where tolerance protocols and genotyping are well-established, to describe any relationship they may see.

Flora Peyvandi of Milan collected multi-national data on patients with **afibrinogenemia or hypofibrinogenemia**. The most frequent sites of bleeding were the mucosa (40.4% of hemorrhages), joints (23%), muscles (16%), digestive tract (15%), epistaxis 10%, menorrhagia in women. Central nervous system bleeding was infrequent, as was thrombosis.

Ophira Salomon of Israel retrospectively reviewed bleeding at **164 deliveries** in 62 women with homozygous or doubly-heterozygous **factor XI deficiency**. Prophylaxis, if given, consisted of fresh-frozen plasma. Of 146 vaginal deliveries, 132 were managed without and 14 with plasma infusion. Excessive bleeding occurred in 32/132 (24%) despite plasma and in 2/12 (17%) not given plasma. For Caesarian section, 12 were prepared with plasma but two of them had excessive bleeding nevertheless, and 6 others had no prophylaxis and none had excessive bleeding. Hemorrhaging occurred in 2/14 women (14.3%) with II/II genotype, 12/32 (37.5%) with III/III genotype, and 3/13 (23.1%) with III/III genotype. These figures were not statistically significant. The women were not segregated according to prior history of bleeding in themselves or family members (but that might not be reliable, childbirth can come early, before much of a history is established.) Given that some patients with fairly severe factor XI deficiency rarely hemorrhage, I am not surprised that many women gave birth, even had Caesarians, without excessive bleeding. On the other hand, it's disturbing to see that nearly a quarter of the women who did receive prophylactic plasma still hemorrhaged. For some women with severe factor XI deficiency, plasma prophylaxis may not be enough. Additional plasma or factor XI concentrate ought to be on hand.