

## **Progress in the Molecular Biology of Inherited Bleeding Disorders**

Chair: David Lillicrap, Department of Pathology & Molecular Medicine, Queen's University, Kingston, ON, Canada

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### *Overview*

David Lillicrap, Canada

The molecular basis of hemophilia has been known for more than 20 years, since the characterization of factor VIII (FVIII) and factor IX (FIX) in the mid-1980s, Dr. David Lillicrap said. This knowledge was expected to dramatically improve hemophilia care through a greater understanding of pathogenesis, better diagnosis, and the development of more optimal therapies. While our understanding of the pathogenesis and ability to diagnose hemophilia have drastically improved, therapeutic advances have been a little slower in coming than originally hoped. Recently, however, innovations and advances in molecular biology have shown real promise.

### *Coagulation Proteins with Enhanced Biological Properties*

Steven Pipe, Department of Pediatrics and Communicable Diseases, University of Michigan, U.S.A.

Advances over the past two decades have resulted in a gradual but significant improvement in clinical care for people with bleeding disorders, Dr. Steven Pipe said. Diagnostic improvements, along with factor replacement therapies and prophylaxis, have resulted in improved survival, the prevention of joint damage, inhibitor management, and growing advocacy for global improvement in hemophilia care.

The tools available have progressed from pathogen screening, to viral inactivation for plasma-derived products, to recombinant technology, Dr. Pipe said. "Now it is possible to build on the platform of recombinant DNA technology using bioengineering strategies as tools to further advance factor replacement and move ever closer to an ultimate cure."

One of the greatest challenges posed by current therapies is the prohibitive cost of recombinant products, particularly for prophylaxis. Improving FVIII secretion efficiency could provide more and cheaper treatment products. Among the strategies currently under development:

- B-domain deleted FVIII has shown a dramatic (17-fold) increase in messenger RNA levels, but only a 30% to 50% increase in secretion efficiency.
- Use of a modified B-domain variant has resulted in higher potency FVIII, which has a greater hemostatic effect, requiring much lower doses.
- It is also possible to improve FVIII RNA expression and discourage its tendency to misfold by using an A1 point mutation. Similar approaches have seen success in enhancing FIX expression.

Dr. Pipe expressed reservations about attempts to use PEGylation (attaching one or more chains of a substance called polyethylene glycol [also known as PEG] to a protein molecule) to improve FVIII half-life. The application of PEG to FVIII increases the size of the protein so that it is too large for the kidney to filter and clear. Though FVIII appears to be cleared by the liver, not the kidney, long-term toxicity is possible as PEG accumulates in tissue over a long period.

Attempts to use PEGylated liposomes or PEGylated von Willebrand factor (VWF) as a carrier for FVIII show promise, however. When FVIII is reconstituted with these carriers, it covalently binds with them. Studies have indicated a 1.5- to 1.6-fold prolongation of activity, with enhanced hemostatic control. Studies have also found a statistically significant increase in the number of bleed-free days compared with the number for standard recombinant formulations. The same approaches are being applied to FIX and rFVIIa, Dr. Pipe said.

“There is a wealth of basic studies from labs around the world identifying important structural advances with FVIII, FIX, and rFVIIa, leading to real preclinical opportunities for product development and some that are moving forward to clinical trials,” Dr. Pipe said. Over the coming years, he predicted a steady development of new bioengineered products and advances in tools that will enhance and expand patient care.

#### *Bringing Hemophilia Gene Therapy to the Clinic*

Kathy High, Children's Hospital of Philadelphia, PA, United States

The goal of gene transfer for inherited disease is to achieve long-term expression of the donated gene at a level high enough to improve or eliminate symptoms of the disease, said Dr. Kathy High. Current treatment for hemophilia relies on the infusion of clotting factor concentrates. However, levels fluctuate significantly after infusion, even during prophylaxis. The goal of gene therapy would be to maintain a consistent level of factor that would prevent bleeds, rather than treating them as they occur.

Hemophilia has many advantages as the target of study in gene therapy:

- A wide therapeutic window (i.e. there is a big difference between the minimum effective dose and the minimum toxic dose)
- Low threshold for therapeutic effect (i.e. you don't need a big increase in factor levels to have positive results)
- Wide prevalence of the disease (compared to many other genetic diseases)
- Availability of mouse and dog models that allow preclinical screening and testing
- Lack of the need to tightly regulate expression (you can't really have too much factor, so you don't need to worry how many copies of the gene are being made)
- Quantitative, rather than qualitative, endpoints that are easily measured (i.e. you can measure the amount factor in the blood to determine the effect of therapy)

All gene therapy has three essential components, Dr. High said: a vector (gene delivery vehicle), the gene of interest (in the case of hemophilia, transgenic FIX, FVIII, or FVIIa), and relevant target tissue.

Several gene transfer trials took place in the late 1990s and early 2000s, using a variety of approaches. In general, Dr. High said, they demonstrated safety but not efficacy. However, they did lay the groundwork for the next generations of studies.

Dr. High gave an overview of the evolution of approved clinical trials that have targeted the liver with an adeno-associated virus (AAV) vector expressing FIX. Long-term expression can be achieved by inserting the vector directly into stem cells, but this is dangerous because of the possibility of mutagenesis (including activating cancer genes). Long-term expression can also be

achieved by inserting a non-integrating vector, such as AAV, into long-lived post-mitotic cells (i.e. cells that are no longer dividing).

After animal trials demonstrated the safety and efficacy of inserting recombinant AAV vectors expressing FIX, the first clinical trials were undertaken, Dr. High said. In these small-scale trials, intramuscular injections were used first. Results suggested that they were safe and well tolerated, and that FIX expression increased. However, circulating levels of FIX did not increase significantly, so no therapeutic benefit was demonstrated. Because it was impractical to inject the vector into enough intramuscular sites to achieve therapeutic levels, attempts were made to transduce larger areas of skeletal muscle by using intravascular delivery. This approach seems more promising and will be of benefit to the large number of hemophilia patients who cannot tolerate liver injections because of the presence of hepatitis.

In preclinical studies, a clear dose advantage has been demonstrated when AAV is injected into the liver. Injections into skeletal muscle tended to provoke unwanted immune responses in dog models, but there was tolerance with liver injections. In the first clinical trials, therapeutic levels of FIX were maintained for four weeks, then began to decline. At the five-week point, however, liver function tests showed an increase that eventually decreased without further treatment. Dr. High postulated that this human-specific side effect was the result of a T-cell mediated immune reaction to the AAV vector.

As a result of this discovery, a new round of gene transfer clinical trials has been approved that will study injection of the AAV-FIX into the liver, with the addition of immunosuppressant therapies to reduce the T-cell response.

Dr. High said that gene transfer studies were showing great promise, and she counselled patience, noting that it has only been 15 years since the first gene therapy studies.

#### *Engineered Liver Cells for the Treatment of Hemophilia*

Kazuo Ohashi, Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Japan

Nine of 11 clotting factors are actually produced by liver cells (hepatocytes), Dr. Kazuo Ohashi said. "There is great potential for using these cells to create longer, more sustained factor activity for people with hemophilia."

He outlined a process of hepatocyte transplantation that has shown positive results in hemophilic dogs. It involves isolating hepatocytes from donors, purifying them to 98% purity, then transplanting them. In a small study of patients with FVII deficiency at King's College Hospital in London, hepatocyte transplant has resulted in prolonged and significant therapeutic benefits.

In mice trials, Dr. Ohashi said, FIX levels increased to 1% after hepatocyte transplant and were sustained for a significant period. In addition, a second transplant 15 days later saw the levels increase higher still, suggesting that increased sustained expression of FIX was possible and that it could be incrementally raised by repeated transplant until the appropriate level was achieved. Moreover, the expression appears to be sustained over longer periods. "This clearly

indicates that hepatocyte transplant therapy is a viable option for the treatment of hemophilia," he said.

Dr. Ohashi outlined the possibilities of locating hepatocytes in other parts of the body outside the liver. This would help address the somewhat transient time frame during which engineered hepatocytes have remained functional, which has been one of the major drawbacks of this approach.

*Cell-Based Strategies for Inherited Protein Deficiencies*

Ali Ugur Ural, Gülhane Military Medical Academy, Ankara, Turkey

Joints are the most frequent sites of bleeds associated with hemophilia, and the associated joint damage is one of the disease's most serious complications, Dr. Ali Ugur Ural said. Hemophilia is characterized by inflammatory synovitis and cartilage destruction. Iron, which is deposited from blood into the joint, also plays a role in the development of hemophilic arthropathy by activating genes involved in synovial proliferation and stimulation of the inflammatory cytokines interleukin-6 (IL-6), interleukin-1 (IL-1), and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ).

Adult mesenchymal stem cells (MSCs) can secrete a broad spectrum of macromolecules that create a regenerative environment for a variety of injured tissues. They also prompt a self-regulated regenerative response. During the development of hemophilic synovitis, however, high levels of IL-1 and TNF- $\alpha$  suppress MSCs, thereby limiting their reparative response.

In mouse studies, Dr. Ural said, those lacking TNF- $\alpha$  receptor 1 formed more cartilage and bone. In another study, intra-articular administration of MSCs resulted in the regeneration of meniscal tissue and slowed the progression of joint disease in osteoarthritis.

Dr. Ural suggested that blocking TNF- $\alpha$  signalling with MSCs in hemophilic synovitis will not only arrest disease progression by limiting inflammation, but could also reinforce the regenerative potential of the MSCs and contribute to the re-establishment of secondary joint hemostasis. However, he said, there is still controversy about the tendency of MSCs to induce cartilage hypertrophy and the role they possibly play in arthritic progression, so extensive animal studies are needed.