Mutagenesis and Expression of Mammalian Clotting Factor IX

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Hemophilia B

- X-linked blood clotting disorder characterized by a deficiency in the factor IX protein
- Factor IX is normally produced in the liver and secreted into the blood at a concentration of 5,000 ng/ml
- The severity of the disease depends upon the location of the mutation within the factor IX gene
- Relatively rare disease affecting 1 in 40,000 people
Clotting Pathway

- Factor IX is involved in the intrinsic pathway
- Factor IXa, calcium ion, phospholipids, and Factor VIII activate Factor X
- Clotting is still capable through the extrinsic pathway
Treatment For Hemophilia B

- Currently the only treatment for Hemophilia B is to infuse factor concentrates at the time of a bleed, however this treatment is expensive and inconvenient.
- Recently, gene therapy has become a feasible cure for hemophilia.
What is Gene Therapy?

• Gene therapy uses some type of vector to deliver the correct copy of a gene to the host cells.

• In this experiment AAV (adeno-associated virus) was used to deliver the correct copy of the factor IX gene to skeletal muscle cells.

• The host cells then produce and secrete the normal factor IX protein into the blood.
### The AAV Transgene

<table>
<thead>
<tr>
<th>ITR</th>
<th>Promoter</th>
<th>FIX</th>
<th>Intron</th>
<th>Factor IX</th>
<th>Poly A Tail</th>
<th>ITR</th>
</tr>
</thead>
</table>

- The transgene located in the AAV DNA expresses the factor IX gene
- Different promoter, intron, and poly A tail combinations can increase expression of Factor IX
Promoter Combinations

• Viral vs. Tissue specific promoters
  – Human Skeletal Alpha Actin (HSA)
  – Human alpha anti-trypsin (HAAT)
  – Cytomegalovirus (CMV)

• It has been found that:
  – HSA constructs expressed poorly in muscle
  – CMV promoter is shut down in vivo in the liver
The Goal of This Experiment

- Create two mutations in the Factor IX gene which are thought to decrease clotting times
- Breed mice to be hemophilic and immunodeficient
- Production of AAV
- Inject mutant human Factor IX constructs into double knockout mice
  - Human factor IX corrects the bleeding defect in mice
  - Mice create antibody to human factor IX protein
The Mutant Constructs

- **K5A**- Previously been shown that FIX has a binding affinity to Collagen IV located in the interstitial space thus decreasing amount of protein that makes it to the blood

- **R338A**- Thought to increase the specific activity of factor IX three fold during its cleavage from factor IX to factor IXa
Mutagenesis of Canine Factor IX

T7 \[\rightarrow\] 2.3 kB \[\rightarrow\] T3

T7

EcoRV

Xho1

lacZ

K9-2 (520 bp) \[\rightarrow\] R338A-1 \[\rightarrow\] R338A-2 (775 bp) \[\rightarrow\] K9-4

SK-1 (290 bp) \[\rightarrow\] K5A-1 \[\rightarrow\] K5A-2 (697 bp) \[\rightarrow\] K9-4

BglII

BamHI

pSK-cF IX (EcoRV/BamHI) 4.8 kB
Breeding Strategy to Create Double Knockouts

**P₁**
- Male: HB Normal / Rag 1
- Female: HB / Rag 1 Normal

**F₁**
- Male: HB / Rag 1 Carrier
- Female: HB Carrier / Rag 1 Carrier

**F₂**
- Male: HB / Rag 1
- Female: HB / Rag 1 or HB carrier / Rag 1

**F₃**
1/2 Double Knockouts
DNA Extractions for Hemophilia B and Rag 1 Genotyping

- Bleed mice from the retro-orbital plexus behind the eye
- Spin down the blood, remove the plasma, and extract DNA from the blood cells
- Use a PCR based strategy to genotype mice for hemophilia and Rag 1 alleles
PCR Strategy Used to Genotype

Normal Rag 1 gene

Rag 1 | Neomycin | Pgk-1 | Rag 1

Rag 1 | Hemophilia B

[Genetic analysis images]
Confirmation of Genotypes

• Rag 1
  – ELISA (Enzyme-Linked Immunosorbent Assay) -measuring IgG in plasma
  – FACS (Fluorescence-Activated Cell Sorter)-
    Stain CD-3 cells, separate, and count

• Hemophilia
  – aPTT (activated Partial Thromboplastin Time)-
    measures the active Factor IX in the blood
Production of AAV

- Grow up 100 plates of AAV free 293 cells (human embryonic kidney cells)
- Infect with adenovirus
- Transfect with desired expression plasmid and a trans plasmid supplying rep + cap proteins
- Harvest cells and lyse by sonication
- Purify by 3 CsCl gradient ultra-centrifugations
- Titer virus with a quantitative slot blot hybridization
PCR Strategy used to locate AAV in CsCl gradient

• AAV usually located around 1.40 g/ml CsCl
• PCR primers located in CMV promoter and intron 1 were designed
• Only 26 cycle PCR was run to quantitate the amount of virus per fraction
Titering of the virus

- Plasmid DNA was serially diluted to 10, 5, 2, 1, and .5 ng
- 2 \( \mu \)l of viral DNA was extracted and loaded as 5, 2.5 and 1 \( \mu \)l aliquots
- Viral titer was \( \sim 10^8 \) viral particles / \( \mu \)l
Injection of Mutant Factor IX
AAV Vector

- Collect blood via a tail cut into sodium citrate and perform an aPTT to prove that mice are truly hemophillic
- Inject AAV intramuscularly
- Inject mononine (plasma derived human factor IX) intraperitoneal to prevent the mouse from bleeding to death after surgery
Mice Numbers for Injection

- Amount of virus injection is based on size (kg) of animal
- 2 mice - $1 \times 10^{11}$ particles of AAV-CMV-hFIX-WT
- 2 mice - $3 \times 10^{11}$ particles of AAV-CMV-hFIX-WT
  - To determine when muscle is saturated with virus
- 3 mice - $1 \times 10^{11}$ particles of AAV-CMV-hFIX-K5A
- 3 mice - $1 \times 10^{11}$ particles of AAV-CMV-hFIX-R338A
Mutant Factor IX Analysis

• K5A
  – Perform an ELISA comparing levels of Factor IX in the blood of mice injected with normal factor IX and mutant factor IX
  – Sacrifice mice and perform immunofluorescence staining of muscle cross sections to show that Factor IX no longer binds to collagen IV

• R338A
  – Perform an ELISA and aPTT on plasma taken from R338A injected mice and normal factor IX injected mice to show that decreased bleeding time correlates with factor IX levels
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