Module 5 Gene Therapy and diseases-I

Lecture 29

Cystic Fibrosis (part I)

29.1 Introduction:

Cystic Fibrosis is an autosomal recessive genetic disorder which impairs the lungs, pancreas, liver and intestine. The disease leads to congestion of lungs due to thick viscous secretion, which takes place as a result of abnormal transport of chloride and sodium across the epithelium. Symptoms of cystic fibrosis often appear in early childhood or during infancy, such as bowel obstruction due to meconium ileus in newborn babies. Exercise is essential for growing children suffering with cystic fibrosis, to release mucus from the alveoli.

The major signs and symptoms of cystic fibrosis include:

- Difficulty in breathing (due to tracheal and lung congestion).
- Accumulation of thick, sticky mucus.
- Frequent lung infections (treated with antibiotics and other medications).
- Sinus infections.
- Despite a normal food intake the growth and weight gain is poor.
- Infertility (males can be infertile due to congenital absence of the vas deferens).
- Salty tasting skin.
- Coughing or shortness of breath.
29.2 Cystic fibrosis transmembrane conductance regulator gene:

- Cystic fibrosis is caused by the mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene found at the q31.2 locus of chromosome 7.
- CFTR gene is relatively large around 230,000 base pairs long and produces a 1,480 amino acid long protein. CFTR protein is a membrane bound glycoprotein with a molecular mass of 170 kDa.
- Different mutations in the CFTR gene is the major cause of cystic fibrosis, one such mutation is due to the deletion of three nucleotides which corresponds to the amino acid phenylalanine (F) located at the 508\textsuperscript{th} position on the protein. This mutation is denoted by \(\Delta F508\) (\(\Delta\) signifies deletion, F is phenylalanine, 508 is the position of amino acid) and accounts for 66-70% cystic fibrosis cases worldwide.
- Normal people have one or two working copies (alleles) of the CFTR gene, only one is needed to prevent cystic fibrosis. Cystic fibrosis develops in individuals who cannot produce a functional CFTR protein. CFTR gene is structurally similar to ATP-binding cassette transporters (ABC) gene and the product of CFTR gene is a CFTR protein.

29.3 CFTR protein:

- CFTR protein is a chloride ion channel involved in creating sweat, digestive juices and mucus.
- It is a 1480 amino acid membrane bound glycoprotein with a molecular mass of 170kDa.
- It belongs to the ABC superfamily of proteins.
- It comprises of a pair of six span membrane bound regions each connected to a nuclear binding factor (NBF) which binds ATP.
- An R-domain is present between these two units which comprises of numerous charged amino acids. Within the ABC superfamily the R-domain is the main characteristic of CFTR protein and has protein kinase A (PKA) and protein kinase C activity.
This channel opens only when the R domain gets phosphorylated by PKA and ATP is bound to the nuclear binding domain (NBD).

**Figure 29.1 Schematic representation of a CFTR protein:**

The mutation in CFTR gene has been classified into five classes:

1. Class I mutation causes no CFTR protein output due to defective synthesis.
2. Class II mutation causes intracellular degradation of CFTR protein due to defective maturation of the protein.
3. Class III mutation causes defect in gating mechanism due to the blocked regulation.
4. Class IV mutation causes decreased chloride movement due to decreased conductance.
5. Class V mutation causes reduced wild-type CFTR protein production.
29.1 Table representing the nomenclature of CFTR mutations:

<table>
<thead>
<tr>
<th>Class</th>
<th>Classification</th>
<th>Mutations involved</th>
<th>Occurrence worldwide</th>
<th>CFTR protein outcome</th>
<th>Severity of Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Defective synthesis</td>
<td>W1282X, G542X</td>
<td>10%</td>
<td>No protein</td>
<td>Severe</td>
</tr>
<tr>
<td>II</td>
<td>Defective maturation</td>
<td>F508del, N1303K</td>
<td>70%</td>
<td>Intracellular Degradation</td>
<td>Severe</td>
</tr>
<tr>
<td>III</td>
<td>Blocked regulation</td>
<td>G551D</td>
<td>3-4%</td>
<td>Gating defect</td>
<td>Severe</td>
</tr>
<tr>
<td>IV</td>
<td>Decreased conductance</td>
<td>R117H, R347P</td>
<td>&lt;2%</td>
<td>Decreased Cl movement</td>
<td>Mild</td>
</tr>
<tr>
<td>V</td>
<td>Decreased abundance</td>
<td>A455E, 3849+10KB C→T</td>
<td>&lt;1%</td>
<td>Reduced wild type CFTR</td>
<td>Variable</td>
</tr>
</tbody>
</table>

29.4 Effect of cystic fibrosis on different organs

- Sinuses: Inflammation of sinuses resulting from infection (sinusitis).
- Lungs: Build-up of thick, sticky mucus in the lungs resulting in bacterial infection and widened airways.
- Skin: Production of salty sweat by the sweat glands.
- Liver: Blockage of biliary ducts takes place.
- Pancreas: Blockage of pancreatic duct takes place.
- Intestines: Unable to fully absorb the nutrients.
- Reproductive organs: Absence of vas deferens in male.
29.5 Pathogenesis of Cystic Fibrosis:

As a result of mutation in the long arm of chromosome number 7 the CTFR protein can no longer function properly leading to deranged or unbalanced transport of chloride, sodium and bicarbonate ions. This unbalanced transport of CFTR-affected ions leads to increased sodium and chloride content in sweat and increased resorption of sodium and water from the respiratory epithelium causing mucostasis characterized by relative dehydration of the airway epithelium and alteration in the tenacity and viscosity of mucous and ciliary dysfunction.

Figure 29.2 Mechanism of cystic fibrosis pathogenesis:

Mucostasis causes less washout of pathogenic organisms and blockage of airway leading to chronic infection, recurrent bronchiectasis and other manifestations of cystic fibrosis. The defect in the CFTR protein also causes disorders in the gut epithelium, pancreas, liver and reproductive tract as mentioned above.
29.6 Gene Therapy for Cystic Fibrosis

Drug trial has been categorized into five phases as shown in the flowchart of drug trial phases below (figure 29.3).

The phase 2 and most phase 3 drug trials are currently designed as randomized, double-blind, and placebo-controlled.

**Randomized:** In the randomized design each study subject is randomly assigned to receive either the study treatment or a placebo (fake treatment).

**Blind:** In this form of study targeted individuals do not know about the treatment. In case of double-blind study the clinician also do not know the kind of treatment being given to the targeted individuals. The purpose of 'blinding' is to prevent biasness, since if a clinician knew about the treatment regime, he/she might be tempted to manipulate the study. Moreover, a clinician might be biased in sampling and analyzing the data in order to show the higher efficacy of the tested gene therapy product.

There is another form of double-blind design known as the “double-dummy” design which provides additional security against biasness or placebo effect. In this design the patient is administered with both placebo as well as active doses alternatively during the study period.
**Placebo-controlled:** The placebo helps the researchers to differentiate the effect of the study treatment from the placebo effect and effectively analyze the case.

**Figure 29.3 Different phases of drug trial:**

[Diagram showing different phases of drug trial: PHASE 0, PHASE 1, PHASE 2, PHASE 3, PHASE 4, with stages like PHARMACODYNAMICS AND PHARMACOKINETICS, SCREENING FOR SAFETY, ESTABLISHING THE TESTING PROTOCOL, FINAL TESTING, POSTAPPROVAL STUDIES.]

Most PHASE 2 and PHASE 3 drug trials are designed as:
- Randomized
- Double-blind
- Placebo controlled
Lecture 30

Cystic Fibrosis (part II)

30.1 Vectors used for gene therapy clinical trials

Vectors used for cystic fibrosis gene therapy clinical trials include viral vectors as well as non-viral vectors. Viral vectors generally used are adenovirus and adeno-associated virus (AAV). General route of the vector delivery includes aerosol mediated delivery via the nasal epithelium and lungs. Adenovirus at high dose is associated with side effects like transient fever, myalgia, flu-like symptoms, transient and local inflammation. Adeno-associated virus serotype 2 (AAV2) is associated with lesser and tolerable possible side effects less severe than that developed by adenovirus.

During the clinical trials the change in nasal potential difference (NPD), CFTR mRNA, CFTR protein, and forced expiratory volume in 1 second (FEV1) were measured. Due to increased luminal sodium absorption, individuals suffering from cystic fibrosis disease have a significantly more negative nasoepithelial surface than normal. To determine the function of the lung the amount of air which can be forcibly exhaled from the lungs in the first second of a forced exhalation is measured using spirometry.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Delivery route(s)</th>
<th>Safety concerns/Side effects</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoviral vector</td>
<td>Nose</td>
<td>No</td>
<td>NPD: decreased baseline towards normal value</td>
<td>Zabner et al., Cell. 1993</td>
</tr>
<tr>
<td></td>
<td>Nose-Lung</td>
<td>Yes, Transient inflammation at</td>
<td>NPD: variable</td>
<td>Crystal et al., Nature</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CFTR mRNA:</td>
<td></td>
</tr>
</tbody>
</table>

Table 30.1 Various viral vector gene therapy clinical trials conducted:
<table>
<thead>
<tr>
<th>Gene Therapy Vector</th>
<th>Location</th>
<th>Effect at Highest Dose</th>
<th>Cystic Fibrosis Gene Expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoviral vector</td>
<td>Nose</td>
<td>Local inflammation at highest dose</td>
<td>NPD: unchanged CFTR and mRNA: detectable mostly at higher doses</td>
<td>Knowles et al., NEJM. 1995</td>
</tr>
<tr>
<td>Adenoviral vector</td>
<td>Nose</td>
<td>No</td>
<td>NPD: partial correction</td>
<td>Hay et al., Human Gene Therapy. 1995</td>
</tr>
<tr>
<td>Adenoviral vector</td>
<td>Nose</td>
<td>No</td>
<td>NPD: mild normalization</td>
<td>Zabner et al., J Clin Inv. 1996</td>
</tr>
<tr>
<td>Adenoviral vector</td>
<td>Nose-Lung</td>
<td>No</td>
<td>CFTR mRNA and protein: some detected in nose &gt; lung</td>
<td>Bellon et al., Human Gene Therapy. 1996</td>
</tr>
<tr>
<td>Adenoviral vector</td>
<td>Lung</td>
<td>No</td>
<td>CFTR mRNA: partially positive but reduced in subsequent administration</td>
<td>Harvey et al., J Clin Inv. 1999</td>
</tr>
<tr>
<td>Adenoviral vector</td>
<td>Lung</td>
<td>Flu-like symptoms</td>
<td>Vector DNA: detectable</td>
<td>Zuckerman et al., Human Gene Therapy. 1999</td>
</tr>
<tr>
<td>AAV2</td>
<td>Nose</td>
<td>No</td>
<td>NPD: partial correction</td>
<td>Wagner et al., Laryngoscope.</td>
</tr>
<tr>
<td>AAV2</td>
<td>Lung</td>
<td>Possible</td>
<td>CFTR mRNA: none detected</td>
<td>1999</td>
</tr>
<tr>
<td>------</td>
<td>-------</td>
<td>----------</td>
<td>--------------------------</td>
<td>------</td>
</tr>
<tr>
<td>AAV2</td>
<td>Nose</td>
<td>No</td>
<td>mRNA: none detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Aitkin et al., Human Gene Therapy. 2001</td>
<td></td>
</tr>
<tr>
<td>AAV2</td>
<td>Nose-Lung</td>
<td>Well tolerated, possible side effects reported</td>
<td>NPD: no change No change in histology of IL-8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wagener et al., Human Gene Therapy. 2002</td>
<td></td>
</tr>
<tr>
<td>AAV2</td>
<td>Lung</td>
<td>No</td>
<td>FEV1: Improvement trend CFTR mRNA undetectable</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moss et al., Chest. 2004</td>
<td></td>
</tr>
<tr>
<td>AAV2</td>
<td>Lung</td>
<td>No</td>
<td>FEV1, sputum markers, antibiotic days: No improvement</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moss et al., Human Gene Therapy. 2007</td>
<td></td>
</tr>
</tbody>
</table>

### 30.2 Gene therapy trial using viral vector

30.2.1 Objectives:

- Determine the safety and tolerability of repeated doses of aerosolized AAV2 vector encoding the complete human CFTR cDNA.
- Evaluation of pulmonary function, lung abnormalities, cytokines, vector shedding, serum neutralizing antibody, and expression of transgene in patients.

30.2.2 Method:

Cystic fibrosis patients with mild lung disease were selected for eight different cystic fibrosis centers in the United States. Subjects were given a dose of $1 \times 10^{13}$ recombinant adeno-associated virus containing CFTR gene nalong with appropriate control.

30.2.3 Observation:

No adverse effects or laboratory abnormalities were noted between the treatment groups. Vector shedding in sputum was observed in the patients. All subjects receiving CFTR gene exhibited an increase in serum AAV2-neutralizing antibodies.

30.2.4 Conclusions:

From this clinical trial it was concluded that the repeat doses of aerosolized AAV2 containing CFTR gene were safe and well tolerated. It resulted in encouraging improvement in pulmonary function in patients with cystic fibrosis.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Delivery route(s)</th>
<th>Safety concerns/Side effects</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CFTR mRNA: not detected</td>
<td></td>
</tr>
<tr>
<td>DC-Chol/DOPE</td>
<td>Nose</td>
<td>Possible</td>
<td>NPD: partial correction</td>
<td>Gill et al., Gene Therapy. 1997</td>
</tr>
<tr>
<td>DC-Chol/DOPE</td>
<td>Nose</td>
<td>No</td>
<td>NPD: partial correction CFTR mRNA: detected in some</td>
<td>Hyde et al., Gene Therapy. 2000</td>
</tr>
<tr>
<td>DOTAP</td>
<td>Nose</td>
<td>No</td>
<td>NPD: partial correction CFTR mRNA: detected in some</td>
<td>Proteus et al., Gene Therapy. 1997</td>
</tr>
<tr>
<td>EDMPC</td>
<td>Nose</td>
<td>No</td>
<td>NPD: unchanged CFTR mRNA: undetectable</td>
<td>Noone et al., Molecular Therapy. 1999</td>
</tr>
<tr>
<td>GL67 vs naked pDNA</td>
<td>Nose</td>
<td>No</td>
<td>NPD: significant response with both vectors CFTR: not detected</td>
<td>Zabner et al., J Clin Invest. 1997</td>
</tr>
<tr>
<td>DNA Nanoparticles</td>
<td>Nose</td>
<td>No</td>
<td>NPD: partial correction CFTR DNA: Detectable in treatments and</td>
<td>Konstan et al., Human Gene Therapy. 2004</td>
</tr>
</tbody>
</table>
30.3 Gene therapy trial using nonviral vector

A clinical trial, where compacted DNA nanoparticles were administered to the nasal mucosa of cystic fibrosis subjects to check for the reconstitution of CFTR protein, was conducted by Konstan et al., (P.S Compacted DNA nanoparticles administered to the nasal mucosa of cystic fibrosis subjects are safe and demonstrate partial to complete cystic fibrosis transmembrane regulator reconstitution. Konstan MW, et al. Hum Gene Ther. 2004 Dec;15(12):1255-69. Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA.)

30.3.1 Method:

Subjects with age greater than 18 years having FEV1 more than 50% and nasal potential difference (NPD) isoproterenol response ≥-5mV were allowed to enter in this study.

Some facts:

It is to be noted that the forced expiratory volume in 1 second (FEV1) > 50% and NPD ≥-5mV is the characteristic feature of classical cystic fibrosis.

The study subjects were subjected to a double-blind, dose escalation gene transfer trial. Placebo (saline) or compacted DNA was superfused or administered onto the inferior turbinate of the right or left nostril. Using the polyethylene glycol substituted 30-mer lysine peptides the plasmid DNA carrying the CFTR gene was compacted into DNA nanoparticles. These DNA nanoparticles were then administered through the nasal mucosa. The study was conducted for three dose levels (DLI, DLII, DLIII).
Twelve subjects were enrolled for the study: 2 in dose level I (DLI) (0.8 mg DNA was administered), 4 in DLII (2.67 mg DNA was administered), and 6 in DLIII (8.0 mg DNA was administered). The primary trial assessment end points were safety and tolerability, and secondary trial assessment end point was gene transfer. Along with the primary and secondary trial assessment the routine clinical assessments and laboratory tests were performed. The subjects were serially evaluated for serum IL-6, complement, and C-reactive protein. The nasal washings were taken for cell counts, protein, IL-6, and IL-8. The pulmonary function and hearing tests were also performed.

30.3.2 Observation:

Following the tests conducted on the subjects

- Occurrences of no serious adverse events took place, and no events were attributed to compacted DNA.
- Association of serum or nasal washing inflammatory mediators with administered compacted DNA was not observed.
- On day 14, vector polymerase chain reaction (PCR) analysis showed a mean value in DLIII nasal scraping samples of 0.58 copy per cell.
- In the eight study subjects partial to complete NPD isoproterenol responses were observed in one of the two test subjects in DLI, three of the four test subjects in DLII, and four of the six test subjects in DLIII.
- After gene transfer the corrections persisted for as long as 6 days in almost all the subjects, in one of the subjects the correction persisted for 28 days.

From this study it was concluded that the compacted DNA nanoparticles can be safely administered to CF subjects through nasal mucosa, vector gene transfer was confirmed and partial NPD correction was observed.
Lecture 31

Duchenne Muscular Dystrophy

31.1 Introduction

One out of 3500 boys is born with the Duchenne muscular dystrophy (DMD). Females are carrier for this disease. This hereditary disease occurs due to the absence or very less expression of dystrophin protein, which acts as an important structural component present within the muscle tissue and provides stability to the dystroglycan complex structure (DGC) of the cell membrane. As a result of mutation or impaired function of dystrophin gene, dystrophin protein synthesis gets impaired hence non-functional dystrophin protein is formed causing the DMD. The credit of identification of this gene goes to Louis M. Kunkel. He identified the gene in the year 1987 after the reported discovery of the mutated gene causing Duchenne muscular dystrophy (DMD) in 1986. The DMD is a recessive X-linked disease which occurs due to frameshift mutation in the dystrophin gene. An allelic counterpart of the DMD is the Becker's muscular dystrophy (BMD). The severity of BMD is less as compared to DMD and the appearance of symptoms is later than in DMD. The person affected with BMD generally loses their ability to walk at the age of around 30 years. In DMD dystrophin protein is absent whereas in BMD partially functional dystrophin protein is synthesized that is why BMD is less severe than DMD.

31.2 Dystrophin gene

The dystrophin gene is located on the short arm (p arm) of the X chromosome at the band 2, sub-band 1 and sub-sub-band 2 (Xp21.2). It is the longest known human gene and has a size of 2,400 Kbp approximately 0.1% of the human genome. The transcription of the dystrophin gene takes around 16 hours. The transcribed mature mRNA after splicing is about 14 Kbp. There are about 79 exons (18 isoforms) which code for the dystrophin protein. The dystrophin protein consists of about 3500 amino acid and has a molecular weight of 427KDa.
31.3 Dystrophin protein

Dystrophin (a rod-shaped cytoplasmic protein) is a key player that connects the cytoskeleton of each muscle fiber to the basal lamina, with the help of a protein complex (made of numerous protein subunits more than 50), and thus provides mechanical stability by anchoring and supporting the sarcolemma. The dystrophin protein is made of three domains with different functionalities.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal domain</td>
<td>It is homologous to alpha-actinin and binds to F-actin.</td>
</tr>
<tr>
<td>Rod shaped domain</td>
<td>It is made of 24 triple helical coiled-coil repeats (each comprises of 122 amino acid residues).</td>
</tr>
<tr>
<td>C-terminal domain</td>
<td>This domain is rich in cysteine and contains binding sites for syntrophins, β-dystroglycans and F-actin proteins.</td>
</tr>
</tbody>
</table>

The dystrophin glycoprotein complex (DGC) also called costamere, acts as a bridge between the cytoskeleton, plasma membrane and the basal lamina. The dystroglycan is made of two subunits (α, β). The α-dystroglycan binds to laminin-2 (merosin) protein whereas the β-dystroglycan binds to the cysteine rich region of dystrophin. Sarcoglycans bind to the biglycans and the collagen and forms a tight complex. Syntrophins (peripheral membrane protein) bind to the C-terminal region of the dystrophin. The structure of DGC has been illustrated in the figure 31.1.

The absence of dystrophin results in excess calcium permeation to the sarcolemma (cell membrane). Due to the aberration in these signaling pathways, water enters into the mitochondria causing it to burst. This results in mitochondrial dysfunction causing an increase in stress-induced cytosolic calcium signals. As a result of amplification of stress-induced cytosolic calcium signals the production of stress-induced reactive-oxygen species (ROS) takes place. Following the ROS...
formation, the cascade activation of various complex pathways (not clearly understood) takes place and there is an upregulation of oxidative stress within the cell that causes damage to the sarcolemma and cell death. A large number of cells die as a result muscle fibre necrosis can be observed in biopsy. The dead muscles are replaced with adipose and connective tissue.

**Figure 31.1 Schematic representation of dystrophin biology:**

![Figure 31.1 Schematic representation of dystrophin biology](image)

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Schematic representation of the Dystrophin complex network:

Dystrophin is an important constituent of the costameres and enables connection of the Z discs (present in the sarcomere) to the sarcolemma. Dystrophin plays an important role in providing mechanical stability to the muscle cells during the process of muscle contraction. Dystrophin network constitutes of a number of proteins of which almost 50 have been reported. Some of those proteins have been shown in this figure like the dystroglycans, syntrophins, dystrobrevin, sarcospan, desmin, laminin, syntrophins, sarcoglycan and nitric oxide synthase (N-NOS). Some other proteins associated with the dystrophin complex network have been identified and include agrin, telethonin, dyserin, neurexin, myotonin, fukutin, equatorin, spectrin, calpain, desmusin, integrins etc. Some of the functionalities of the proteins of the dystroglycan network is yet to be understood.

In the absence of dystrophin, the dystrophin complex becomes unbalanced and the components of the complex gets disturbed. As a result of this unbalance the dystroglycans, the sarcoglycans and sarcospan disappear or get completely reduced. Each protein of the dystrophin complex is coded by its own gene which may get impaired as a result of mutations leading to 13 different types of limbgirdle diseases. 5 types of congenital muscular dystrophies and upto 8 types of neuromuscular diseases.
31.4 Symptoms of DMD

The symptoms of DMD can be seen in early childhood days (before age 6) and sometimes during infancy. The characteristic symptom of DMD includes delay of motor neuron activity which independently affects sitting and standing ability. Due to delay in achieving control over the muscle movement the child affected with DMD takes longer time to start walking as compared to normal children. The mean age for walking in boys with DMD has been recorded to be about 18 months. Muscle weakness of the legs and pelvis region progresses with age and the loss of muscle mass also known as muscle wasting takes place. The enlargement of calf muscle occurs followed by the replacement of the muscle tissue by fat and connective tissue generally termed as the “pseudohypertrophic condition”. Weakness in the muscle results in a characteristic way of walking called as “waddling gait”. Due to the tightening of tendons around the heels, the person has to walk on tiptoes resulting in “lordosis” (development of forward curvature of the spine). The affected person finds difficulty in walking and climbing stairs. The muscles of the lower half of the body weakens earlier and more severely than the muscles of other parts of the body like the shoulder, arms, neck etc. In the later stages the affected person is unable to walk (gets confined to the wheelchair) followed by “scoliosis” (curvature of spine to the sides) and “contractures” (tightening of joints). In the last stages of the disease weakening of chest muscle and heart muscle takes place resulting in severe respiratory problem. Death takes place due to heart failure (cardiomyopathy) or respiratory failure.

31.5 Diagnosis of DMD

DMD can be diagnosed by the symptoms as well as by measuring the amount of creatinine phosphokinase enzyme level in the blood serum. This enzyme is involved in muscle degeneration and an elevated level of this enzyme (around 20-100 times more than the normal level) is indicative of DMD. Other tests to confirm DMD includes muscle tissue biopsy, electromyography (EMG) and molecular tests.
31.6 The mdx mouse

To understand the underlying mechanism involved in the process of muscle degeneration and regeneration, the animal model being used is the mdx mouse. The dystrophic mdx mouse contains a point mutation that causes a change in the codon in the dystrophin gene resulting in changeover of glutamine amino acid to termination codon (CAA, CAG encodes for glutamine which may be changed by replacement of any of the three letters with T. In case the C in CAA and CAG is replaced by T then it becomes TAA and TAG, which are stop codons and result in premature stop of dystrophin synthesis and formation of non functional dystrophin in the muscle). All experiments related to DMD use the mdx mouse as the animal model. From the beginning, different stages of skeletal muscle degeneration and subsequent regeneration can be observed in the mdx mouse. As the mdx mouse grows old generally the diaphragm muscle weakens followed by development of fibrosis in the muscle.

31.7 Treatments for DMD

Since the size of dystrophin gene is large, it is susceptible to random mutations. It has been observed that almost two third DMD cases are due to deletion mutation within dystrophin gene whereas one third DMD cases are due to nonsense point mutation which affects the reading frame of the transcript. As a result of nonsense point mutation truncated and non-functional dystrophin protein is formed. Till date no cure for DMD has been found. Thus, the current therapies aim at increasing the quality of life of the patient by controlling the symptoms. Pharmacological strategy involves:

a) The use of a cocktail of drug like prednisone and deflazacort or prednisone and cyclosporine-A. Prednisone is a corticosteroid, believed to delay muscle degeneration by suppressing the individual’s immune system and inhibiting muscle proteolysis. Prednisone also enhances the synthesis of utrophin which is an analogue of dystrophin. The administration of 0.75mg of prednisone (per Kg of the individual per day) increases working of the muscle by two to three years. Side effects of this drug include osteoporosis, high blood pressure, gastro-intestinal pain, cataract, mood swings and excess weight gain.
b) Administration of monoclonal antibodies against myostatin (limits muscle growth) in mdx mouse has shown increased, better muscle functionality and decreased muscle degeneration (low creatinine phosphokinase production). This method needs to be further tested before use in human clinical trials.

31.8 Gene therapy for DMD includes

31.8.1 Transfer of new dystrophin gene: Transfer of new dystrophin gene uses high capacity gutless adenovirus (into the nuclei of dystrophic muscle cell). Since the size of the dystrophin cDNA is very large (14Kbp) and in addition the size of the vector (adenovirus) is also large thus it cannot easily enter the mature myofibre. The myofibre also lacks adenovirus receptors. Adeno-associated virus (AAV) has a smaller size and can be used instead of adenovirus. Limitation of using AAV is that it cannot carry the dystrophin cDNA (since size of dystrophin cDNA is very large). Thus, the truncated cDNA has to be used. It is known that the dystrophin protein consists of N-terminus, C-terminus, cysteine rich region and central rod region. Cysteine rich region, N and C terminus are essential for maintaining the integrity of the DGC thus; the truncated cDNA may be shortened at the central rod shaped domain while retaining the whole N-terminus, C-terminus, cysteine rich region and some portion of the central rod region. The reduced cDNA produced as a result of deleted portions of central rod region is known as the dystrophin mini-gene. The dystrophin mini-gene can be easily inserted into the AAV which has good transfection efficiency as a result the mini dystrophin gene can be successfully targeted to the muscle cells. To overcome the lack of receptors for adenovirus on the myofibre, monoclonal antibodies with specific binding at one end for the adenovirus and another end to various receptors (like integrin) on myofibre cells can be used.

31.8.2 Complete gene as DNA: The whole dystrophin cDNA can be inserted into the plasmid and injected into the blood stream. The limitation of this method is low transfection efficiency and high dose requirement for successful transfection. Electroporation, ultrasound as well as non-viral vectors may be used for delivery of the target gene to the target tissue.

31.8.3 Chimeraplasty: Chimeraplasty utilizes the endogenous repair mechanism, which may be used for repairing the defective dystrophin gene.
31.8.4 Supressing premature termination: Gentamycin, an amino glycoside, has been reported to suppress the premature termination (due to stop codon generated as a result of mutation) and enable the synthesis of 20% functional dystrophin protein in mdx mouse. Its efficacy in human subjects has not been ascertained till date.

31.8.5 Exon skipping: Exon skipping is another technique which may be employed to elevate the effects of DMD by making it milder like BMD. In DMD dystrophin is absent as ribosome encounters premature stop codon thus by skipping the mutated exon the rest of the shorter functional truncated dystrophin can be synthesized. The purpose of exon skipping is to induce the splicing mechanism and remove the introns from the pre mRNA and exons before and after the point mutation or deletion and thus enable read through the rest of the exon as in normal condition.

In order to perform exon skipping antisense oligoribonucleotides (AONs) have to be inserted into the target cell. The AON should target either the exon splice enhancer (ESE) region or the introns-exon splicing region lying within the particular exon that has to be skipped in the pre mRNA. On binding the AON blocks the normal formation of spliceosome preventing the splicing of the exon correctly. Thus the exon skipping takes place. As a result of exon skipping (exons are lost) shorter mRNA, and truncated dystrophin protein is synthesized. In case the skipped exon is not critical and belongs to the central region then the truncated protein can function and stabilize the muscle cell membrane.

The method of production of AONs in vivo involves the modification of the component of spliceosome (U7-snRNAs) which enables the recognition of target binding site rather than the wild type binding site. The U7-snRNAs function by binding to the splice sites at specific regions in the pre mRNA thus impeding the splicing of the specific exon. As a result several protein of varying length is produced from a single gene. The genetic alteration in the U7-snRNA would enable the binding to the splice region of the exon in the dystrophin gene. In this way the exon skipping would be easily performed in the dystrophin gene.
31.9 Clinical studies

1. Romero NB et al., in the year 2004 conducted phase-I study of dystrophin plasmid-based gene therapy in Duchenne/Becker muscular dystrophy. Nine study subjects affected with DMD/BMD were registered for the trial and were injected with full length human dystrophin gene incorporated in the plasmid. The dose administered was either once with 200 or 600µg of DNA or twice, 2 weeks apart, with 600µg of DNA by injection in the radialis muscle. After 3 weeks muscle biopsy, of the region injected with the DNA, was performed and in all study subjects the vector was found in the muscle at the site of injection. Out of the nine, 6 patients were reported to express dystrophin based on the immunohistochemistry and nested RT-PCR analysis. The level of expression was found to be low with 6% complete sarcolemmal dystrophin staining and about 26% partial sarcolemmal labeling. Neither cellular, humoral anti-dystrophin response nor any side effects were observed. Thus, based on the results obtained, it was understood that the exogenous dystrophin expression could be achieved in DMD/BMD subjects after intramuscular transfer of plasmid, without any side effects. This study directs the way for future developments in hereditary muscle dystrophy gene therapy.

2. Van Deutekom JC et al., in the year 2007 demonstrated local dystrophin restoration using AON-PRO051. They induced specific exon skipping during messenger RNA (mRNA) splicing using antisense compounds and corrected the open reading frame (ORF) of the DMD gene and restored dystrophin expression in vitro and in animal models in vivo. They also analyzed the safety, adverse-event profile, and local dystrophin-restoring effect of a single, intramuscular dose of an AON-PRO051, in patients with this disease. Four study subjects were selected on the basis of their mutational status, muscle condition, and positive exon-skipping response to PRO051 in vitro. A dose of 0.8 mg of PRO051 was injected into the tibialis anterior muscle. After 28 days of injection muscle biopsy was performed. Safety measures were taken during the study. The composition of mRNA, and dystrophin expression were assessed. It was observed that PRO051 injection did not cause any adverse effect. Specific exon skipping occurred in each study subject in 64-97% muscle cells. Amount of dystrophin protein estimated ranged from 3 to 12% as compared to the control specimen. 17 to 35% range of quantitative ratio of dystrophin to laminin
alpha2 was estimated as compared to the control specimen. This study indicates that intramuscular injection of the AON-PRO051 resulted in dystrophin synthesis in four patients with DMD who had mutations in the dystrophin gene. This study clearly shows the feasibility of further exploration of the trial with better strategies.

3. In 2012 Bowles DE et al., conducted the first phase 1 gene therapy trial for DMD using a translational optimized AAV vector (capsid variant designated AAV2.5) derived from a rational design strategy. The vector AAV2.5 was generated from the AAV2 capsid with five mutations from AAV1. This novel vector has two benefits first it combines the improved muscle transduction capacity of AAV1 with reduced antigenic cross-reactivity against both parental serotypes and second it retains the AAV2 receptor binding capacity. A randomized double-blind placebo-controlled phase I clinical study was conducted in DMD boys. One group of study subjects were injected with AAV2.5 vector into the bicep muscle in one arm and with saline control in the other arm. Another group of study subjects were injected with AAV empty capsid instead of saline in order to distinguish an immune response to vector versus mini-dystrophin transgene. Muscle biopsy analysis revealed the presence of recombinant AAV genomes in all study subjects with up to 2.56 vector copies per diploid genome. No cellular immune response was observed against AAV2.5 capsid. This study confirms that rationally designed AAV2.5 vector was safe and well tolerated and forms the basis of designing AAV vectors that best suit the clinical objective (e.g., limb infusion gene delivery).

31.10 Challenges faced by gene therapy

- Immune rejection of the foreign protein, plasmid and DNA by the target cells.
- Localization of the desired gene product.
- Insertional mutagenesis risk due to use of viral vectors since the viral vector genome may integrate with the host genome.
**Figure 31.2 Supplementary diagram of ribozyme mediated trans-splicing:**

The schematic representation of ribozyme mediated trans splicing method has been shown here.

Ribozymes are catalytic RNA capable of cleaving and ligating RNA. Group I intron ribozyme in *Tetrahymena* catalyses its own self splicing. The splicing reaction involves two consecutive trans-esterification reactions catalysed in the presence of divalent cation. An endogenous guanosine attacks the 5' splice site resulting in a 5'exon having a 3'OH group. This 5' exon attacks the 3' splice site resulting in the excision of the intron and the ligation of the exon.

These ribozymes can be constructed to carry the correct portion of the gene and replace the correct segment in the defective gene as depicted in this simplified diagram. Thus it can be employed for gene repair.
Lecture 32

Bleeding Disorders (part I)

32.1 Introduction

Blood is a fluid connective tissue and consists of blood cells and various factors suspended in the plasma. It is responsible for circulation of oxygen, nutrients throughout the body and removal of toxic metabolites by transporting them to the excretory system. If blood is allowed to stand in a glass tube it coagulates and if anticoagulants like heparin or chelating agents like ethylenediaminetetraacetic acid (EDTA) is added, it prevents blood coagulation and if kept still for half an hour red blood cells (RBC) form rouleaux (stack of red blood cells) and settle down leaving a clear plasma. On centrifuging the blood at low speed (around 2000 rpm) clear zone of separation between the RBC and the plasma can be seen as a white buffy coat that consists of WBC’s. Contact activation explains the reason behind the coagulation of blood when it is taken from the body and put in a glass tube. The glass surface acts just similar to the collagen and induces similar activation of factor XII and aggregation of thrombocytes (platelet) as a damaged vessel surface. A silicone coating if applied on the glass tube prolongs clotting since its smooth texture reduces the activity of the glass surface.

In this lecture initially, we shall understand the importance and mechanism of blood clotting followed by disorders related to blood and the available remedies.

32.2 Process of blood clotting:

A simplified schematic below depicts the process of blood clotting. During any injury if a blood vessel ruptures then the blood gets exposed to the subendothelial tissue where the plasma enzyme activation cascade begins to function. The endothelium has a unique feature of allowing proper fluidity at normal condition but when it encounters injury, it starts the coagulation process (mediated by various factors and secretions of the endothelial cell) in order to contain the injury and prevent any further loss of blood.
Normally, endothelial cells are not involved in the synthesis of tissue factors but when they encounter cytokines or interact with monocytes they begin to synthesize the tissue factor. The tissue factors are glycosylated intrinsic membrane proteins, which come in contact with blood only when there is any injury. They are located on the adventitious side of the endothelial cells hence do not come in contact with blood under normal condition. There are two classical pathways of blood clotting extrinsic and intrinsic. Since, the tissue factor is an extrinsic protein thus, the clotting initiated by tissue factor falls under the extrinsic pathway. All the blood clotting factors are present in the plasma hence coagulation due to the internal components of plasma falls under the intrinsic pathway.

Figure 32.1 Schematic representations of clotting mechanism:

- Damaged Vessel
  - Blood exposed to subendothelial tissue
  - Plasma Enzyme Activation Cascade
  - Enzyme
  - Prothrombin
  - Thrombin
  - Inactive Plasma protein
  - Inactive Plasma protein

- Simplified schematic representation of blood clotting. The formation of thrombin takes place by two enzyme activations. Thrombin has three important effects: a) generates fibrin b) it activates factor XIII and c) by positive feedback it controls the plasma enzyme activation cascade.
### Table 32.1 The blood clotting factors and their functions involved in the fibrin clot:

<table>
<thead>
<tr>
<th>S.no</th>
<th>Name/ Factor</th>
<th>M.W (kDa)</th>
<th>Protein Type</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fibrinogen (Factor I)</td>
<td>340</td>
<td>Glycoprotein</td>
<td>Adhesive protein involved in clot formation</td>
</tr>
<tr>
<td>2</td>
<td>Prothrombin (Factor II)</td>
<td>72</td>
<td>Vitamin-K dependent</td>
<td>Gets activated to thrombin and acts as a main enzyme in coagulation process</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serine Protease</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Tissue factor or</td>
<td>37</td>
<td>Cytokine receptor</td>
<td>As a lipoprotein initiator of extrinsic pathway</td>
</tr>
<tr>
<td></td>
<td>thromboplastin (Factor III)</td>
<td></td>
<td>class II family</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Calcium ions (Factor IV)</td>
<td>-</td>
<td>-</td>
<td>Coagulation reaction cofactor</td>
</tr>
<tr>
<td>5</td>
<td>Labile factor (Factor V)</td>
<td>330</td>
<td>-</td>
<td>Acts as a cofactor and activates prothrombin to thrombin</td>
</tr>
<tr>
<td>6</td>
<td>Proconvertin (Factor VII)</td>
<td>50</td>
<td>Vitamin-K dependent</td>
<td>Along with factor I initiates extrinsic pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serine Protease</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Anti-hemophilic factor (Factor VIII)</td>
<td>330</td>
<td>-</td>
<td>Acts as a cofactor and activates factor X in intrinsic pathway</td>
</tr>
<tr>
<td>8</td>
<td>Christmas factor (Factor IX)</td>
<td>55</td>
<td>Vitamin-K dependent</td>
<td>In activated form acts as an enzyme for intrinsic factor X activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serine Protease</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Name</td>
<td>Value</td>
<td>Type</td>
<td>Function</td>
</tr>
<tr>
<td>-----</td>
<td>-------------------------------------------</td>
<td>-------</td>
<td>---------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>9</td>
<td>Stuart-prower factor (Factor X)</td>
<td>58.9</td>
<td>Vitamin-K dependent Serine Protease</td>
<td>In activated form acts as an enzyme for the activation of prothrombin in the final common pathway</td>
</tr>
<tr>
<td>10</td>
<td>Plasma thromboplastin antecedent (Factor XI)</td>
<td>160</td>
<td>Serine protease</td>
<td>In activated form acts as intrinsic activator of factor IX</td>
</tr>
<tr>
<td>11</td>
<td>Hageman factor (Factor XII)</td>
<td>80</td>
<td>Serine protease</td>
<td>Involved in starting a partial thromboplastin time (PTT) based intrinsic pathway</td>
</tr>
<tr>
<td>12</td>
<td>Fibrin stabilizing factor (Factor XIII)</td>
<td>320</td>
<td>Transamidase</td>
<td>It cross-links fibrin clot</td>
</tr>
<tr>
<td>13</td>
<td>High-Molecular-Weight Kininogen (Fitzgerald, Flaujeac or William factor)</td>
<td>110</td>
<td>-</td>
<td>Circulates in a complex with factor XI, acts as a cofactor</td>
</tr>
<tr>
<td>14</td>
<td>Prekallikrein (Fletcher factor)</td>
<td>85</td>
<td>Serine protease</td>
<td>Participates in a PTT based intrinsic pathway in the activated form</td>
</tr>
</tbody>
</table>
The intrinsic pathway name is derived from the fact that all the clotting factors required for blood clotting is present “within” the plasma. Let us understand the steps involved in this pathway.

- The first plasma protein involved in the intrinsic pathway is called Hageman factor or factor XII. Factor XII is initially in inactive state but on activation becomes factor XIIa. Factor XII converts to factor XIIa only when it comes in contact with rough surface like the collagen fibres underlying damaged endothelium.
- This process of activation is called contact activation and is a complex process requiring the participation of several other plasma proteins.
- Factor XIIa then catalyzes the activation of factor XI to factor XIa which then causes activation of factor IX to factor IXa.
- Factor IXa then activates factor X to factor Xa which is the enzyme responsible for the conversion of prothrombin to thrombin.
- Another plasma protein factor VIIIa serves as a cofactor (not an enzyme) in the factor IXa mediated activation of factor X.

**The importance of factor VIII in clotting can be understood by the fact that the disease hemophilia which causes excessive bleeding occurs due to the absence of this factor. In few cases, hemophilia has been reported due to an abrupt loss or absence of factor IX.**

Now, let us understand the steps involved in the extrinsic pathway for initiating the clotting cascade.

- As explained in the introduction, this pathway involves an intrinsic membrane protein called the tissue factor. It is not a plasma protein and is located on the adventitious surface or the outer surface of the plasma membrane of various tissue cells, including fibroblasts and other cells in the walls of blood vessels below the endothelium.
- The blood when exposed to the subendothelial cells during vessel damage (causing disruption in the endothelial lining) comes in contact with the tissue factor on these cells which bind to factor VII (a plasma protein). Factor VII is activated to factor VIIa.
• The complex formation between the tissue factor and the factor VIIa takes place on the plasma membrane of the tissue cell. Once the complex is formed it begins the catalytic activation of the factor X.

• Tissue factor and factor VIIa complex also performs the catalytic activation of factor IX, resulting in the catalytic activation of larger quantity of factor X by entering into the intrinsic pathway. As a result amplification in the production of Xa takes place.

In short, by the mechanism studied till now we can assume that clotting begins either by the activation of factor XII to XIIa or by the formation of the tissue factor and factor VIIa complex.

32.3 Common pathway:

The paths, the intrinsic as well as the extrinsic, can be seen to merge at factor Xa as shown in the schematic representation below. This activated Xa begins the catalysis and converts prothrombin to thrombin.

Once thrombin is formed it is believed to be involved in three main functions. (a) Thrombin catalyzes the formation of fibrin, it also contributes to the activation of factors XI and VIII in the intrinsic pathway and factor V, with factor Va then serving as a cofactor for factor Xa. (b) It is involved in the activation of factor XIII. (c) It also controls the plasma enzyme activation cascade by positive feedback mechanism.

Thrombin also activates platelets. For the extrinsic pathway tissue factor is the initiator of clot formation in the body. For intrinsic pathway factor XII is the initiator of blood clotting in the body. Generally the production or formation of, thrombin takes place exclusively by the extrinsic pathway. The amount of thrombin obtained is very less to cause enough stabilized coagulation, still it is sufficient to kick start thrombin’s positive-feedback effects on the intrinsic pathway by the activation of factors XI and VIII and platelets. By this way the intrinsic pathway can be triggered independently of factor XII, and these pathway further result in amplification of thrombin required for adequate coagulation. Thus, the extrinsic pathway is capable of plugging into the intrinsic pathway. Though, the extrinsic pathway synthesizes less amount of thrombin yet it bundles with the intrinsic pathway which has more potential and amplifies the production of thrombin. By coupling with the intrinsic
pathway the extrinsic pathway eliminates the requirement of factor XII as the initiator of clotting mechanism in intrinsic pathway. Indirectly initial thrombin production by extrinsic pathway is capable of eliminating the need for factor XII. Thrombin after recruiting the intrinsic pathway in the mechanism of blood clotting, catalyses the prothrombin to thrombin conversion step on its own by activating factor V and thrombocytes. Indirectly liver is an important player involved in clotting and persons suffering from liver disease mostly have serious bleeding problems. Liver is the organ involved in the production of many of the plasma clotting factors and bile salts which are very important for the absorption of vitamin K in the intestine. The liver requires this vitamin to produce prothrombin and several other clotting factors in order to absorb the lipid-soluble substance vitamin K. The liver requires this vitamin to produce prothrombin and several other clotting factors.

**Figure 32.2 Blot clotting mechanism and its pathways:**
Figure 32.3 Clot formation and biology of fibrin:

This schematic represents the formation of clot due to cross linking of fibrin.

Among the various plasma proteins fibrinogen is the most abundant plasma protein, structurally it consists of Aα, Bβ and γ chain dimers that are connected by disulfide bonds. The fibrinogen dimer is flanked by two D globular domains with a central E domain. It is the most important constituent of the clot and its formation takes place after the fibropeptideA (FpA) and fibropeptideB (FpB) cleavage mediated by thrombin.

Assembly of fibrin monomers into protofilaments (due to non-covalent interaction among monomers) takes place followed by covalent cross linking mediated by transamidase or Factor XIII.
Lecture 33

Bleeding Disorders (part II)

33.1 Hemophilia

Hemophilia is a hereditary recessive X-linked bleeding disorder which mainly affects male individuals since they carry only one X chromosome. Females are carriers of this disease under heterozygous condition and they bear this disease only in recessive homozygous condition. This disorder highly compromises with the blood clotting efficiency of the individual. The individual is unable to clot or coagulate the blood. Clotting of blood is essential to stop bleeding in case of injury.

Three conditions of hemophilia have been defined hemophilia A, hemophilia B and hemophilia C

33.1.1 Hemophilia A is a recessive X-linked genetic disorder, the cause of which is the lack of a functional factor VIII (anti hemophilic factor). Almost 80% of hemophilia cases are of hemophilia A type. This disorder presents itself in 1 out of 10000 male live births. The cause behind hemophilia A is the mutated factor VIII gene.

33.1.2 Hemophilia B is also a recessive X-linked genetic disorder the cause of which is the lack of a functional factor IX (Christmas factor). This disease is also known as Christmas disease. Almost 20% of hemophilia cases are of hemophilia B type. This disorder presents itself in 1 out of 34,000 male live births. The cause behind hemophilia B is the mutated factor IX gene.

33.1.3 Hemophilia C unlike the hemophilia A and B is not a recessive X-linked disorder but it is an autosomal genetic disorder the cause of which is the lack of functional factor XI. Haemophilia C is incompletely recessive because even in heterozygous individuals high amount of bleeding has been reported. The cause behind hemophilia C is the mutated factor XI present on chromosome number 4.
The schematic diagrams below (figure 33.1) may be referred to understand the functionalities of the factor VIII, IX and XI. The implications associated with the mutation in these factors and their impact on the clotting mechanism can thus be understood. This disease causes death of individuals who meet with accidents as well as individuals where internal haemorrhage takes place. This disease needs to be seriously addressed.

**Figure 33.1 Difference between a normal and hemophilic condition:**

When injury occurs and the blood vessel starts leaking out blood then the attachment of platelets at the site of injury enables the vasoconstriction i.e. narrowing of the blood vessel to prevent blood loss. The activation of clotting factors is initiated. Under normal circumstances the blood clotting involves the strong platelet plug formation due to the factor VIII. It stabilizes the fibrin clot and seals the injured tissue thus, stops bleeding. Fibrinolysis is the process used to clear the clot after wound recovery. In haemophilic condition the factor VIII is absent that causes the formation of weak platelet plug. Thus, the wound is not properly sealed hence continuous loss of blood due to leakage through loose fibrin clot takes place. Excessive bleeding may cause death of the individual.
33.2 Various vectors used for gene therapy of hemophilia

Adeno-associated virus (AAV) is the most used virus vector for the clinical trials conducted for hemophilia. This virus is of small size, well known and characterized for its effective gene therapy application. Retroviruses have also been used. Non-viral vectors are also being explored as an alternative to the AAV.

The schematic representation below shows the simplified method of gene therapy strategy being used for the treatment of hemophilia. Here the gene encoding for Factor VIII is inserted inside the viral vector and the human cell is infected with this virus. The gene enters the cell, reaches the nucleus and integrates at specific site. The gene is transcribed inside the cell and the functional factor VIII protein is synthesized which effectively elevates or overcomes the limitations of coagulation of the blood.

Figure 33.2 Gene therapy for haemophilia:
33.3 Animal model for haemophilia

33.3.1 Development of Factor IX deficient mice

A factor IX-deficient mouse model for hemophilia B gene therapy was developed by Wang et al., in the year 1997 to study the bleeding disorder (hemophilia B). The factor IX gene was knocked out and this knock-out mice (−/−) was then comparatively studied with the control wild type (+/+), and heterozygous mice (+/−).

The mice were traumatized by injuring their feet using a mouse restrainer. In case of knock-out mice swollen extremities and hematoma were very prominent whereas, these were found to be reduced in the wild type and heterozygous mice. Using Activated Partial Thromboplastin Time (APTT) assay the factor IX coagulant activity in all three mice wild type (+/+), heterozygous (+/−) and knock-out (−/−) was quantitatively estimated and was found to be 92.1 ± 19.1% for the wild type (+/+), 52.9 ± 10.4% for the heterozygous (+/−) and 5.4 ± 2.9% for the knock-out (−/−) mice.

This study was successful in terms of creating Factor IX knock-out mice.

33.3.2 Development of WAG-F8 (m1Ycb) rats harboring a factor VIII gene mutation

In 2010 Booth et al., developed a rat model for factor VIII in order to study the hemophilia A condition. The main objective of this study was

- The determination of precisely specific factors in the coagulation mechanism in the WAG/RijYcb rats.
- Finding out the genetic defect associated or responsible for the hereditary clotting defect (inherited coagulopathy) in the WAG/RijYcb rats.

The factors responsible for clotting were evaluated and properly assessed for their activity. The animals harboring the mutation were affected and on evaluation of individual clotting factor activities it was found that the affected animals were deficient in a specific factor VIII (FVIII). The FVIII gene is located on the autosome (chromosome number 18) in rats whereas in human and mice it is located on the X chromosome. When the factor VIII cDNA was sequenced it was found that a point mutation, resulting in a substitution of Leucine at 176th position by Proline (L176P),
in the A1 domain of the factor VIII protein had occurred. This mutation is responsible for the disruption of the tertiary structure of the FVIII protein molecule.

When the affected animals were administered with the human plasma or human recombinant FVIII it rectified the coagulation defects.

From this study it can be concluded that this model is unique because the size of the rat is larger as compared to mice and this coagulation defect is present in both sexes. This model can be effectively used to study and develop new therapies for both hereditary as well as the acquired factor VIII deficiency.

33.4 Gene therapy studies on hemophilia

In the year 1993 the stage I clinical trial of gene therapy was conducted by Lu D.R, et al., for hemophilia B without much success. Subsequently, Kay M.A, et al., in the year 2000 reported evidence of AAV mediated factor IX gene transfer and its expression in hemophilia B patients. Roth DA, et al., in the year 2001 conducted a nonviral mediated factor VIII gene transfer in patients with severe hemophilia A. Powell JS, et al., conducted phase 1 trial for severe hemophilia A in the year 2003 using a retroviral construct mediated FVIII gene transfer. The vector containing the construct was administered by peripheral intravenous infusion. Manno CS, et al., in 2006 was able to successfully transduce hepatocytes by AAV mediated factor IX gene transfer. He studied and reported an elevated immune response hampering the gene therapy strategy used by him. Jiang H, et al., in the year 2006 reported expression of factor IX in an individual with severe hemophilia B. In his strategy he used AAV mediated factor IX gene transfer which was administered to the skeletal muscle.

33.5 Adenovirus-associated virus (AAV) vector-mediated gene transfer in hemophilia B

Nathwani et al., conducted a clinical investigation for a new gene therapy in patients with the hemophilia B disorder. A single dose of serotype-8-pseudotyped, self-complementary AAV vector containing a codon optimized factor IX transgene (designated as scAAV2/8-LP1-hFIXco) was administered intravenously through a peripheral vein in six patients suffering with severe hemophilia B. The factor IX activity in patients enrolled in this study was measured to be less than 1%. The
patients were administered low dose, intermediate dose as well as the high dose of the scAAV2/8-LP1-hFIXco vector without any immunosuppressive drugs. The patients were observed for six to sixteen months.

33.5.1 Results

Expression of factor IX increased by 2 to 11% of the normal levels was observed in all study subjects. Out of the 6 study subjects 4 discontinued factor IX treatment and remained free of spontaneous hemorrhage whereas in the other two the duration between prophylactic injections was increased. The study subject out of the two who were administered the high dose of scAAV2/8-LP1-hFIXco vector, one was observed to develop a transient, asymptomatic elevation of serum aminotransferase levels. This elevation was due to the formation of AAV8-capsid-specific T cells in the peripheral blood. The other study subject was observed to develop a small increment in the liver-enzyme levels the cause of this increment was not deduced.

33.5.2 Conclusions

The administration of scAAV2/8-LP1-hFIXco vector via Peripheral-vein infusion caused an enhanced factor IX transgene expression capable of improving the coagulation in study subjects with the bleeding phenotypic trait along with few side effects.
Lecture 34

Tyrosinemia

34.1 Introduction

Tyrosinemia is a genetic disorder which is characterized by the accumulation of the amino acid tyrosine in the blood. Tyrosine is a building block of most proteins. The level of this amino acid in the blood is regulated by tyrosine catabolic pathway, a multistep process that involves a number of enzymes. The cause of Tyrosinemia is the deficiency or shortage of one of the enzymes involved in the tyrosine catabolic pathway.

In untreated condition the tyrosine and its byproducts accumulate in tissues and various organs. Serious medical problems like liver failure, hepatocellular carcinoma, kidney dysfunction, skeletal changes and neurological manifestations are associated with the accumulation of tyrosine and its byproducts in the blood hence, it is important to address this problem and find a suitable treatment for this disease.

34.2 Tyrosine catabolic pathway

- In the presence of phenylalanine hydrolase phenylalanine is converted to tyrosine.
- Tyrosine aminotransferase causes the conversion of tyrosine to 4-hydroxyphenylpyruvate (4HPP).
- 4-HPP is converted to homogentisate (2,5-dihydroxyphenylacetate) in the presence of the enzyme 4-HPP dioxygenase (HPPD) which is an Fe(II) dependent non-heme oxygenase. NTBC (2-[2-nitro-4-(trifluoromethyl) benzoyl] cyclohexane-1,3-dione) or nitisinone is a triketone which inhibits the enzyme 4-HPP dioxygenase (4-HPD) reversibly thus preventing the formation of maleylacetoacetic acid and fumarylacetoacetic acid. As a result, it prevents the formation and accumulation of toxic succinylacetone and succinyl acetoacetate. NTBC is successfully being used to treat type I tyrosinemia patients.
• Homogentisate is oxidized by the enzyme homogentisate oxidase to maleylacetoacetate.
• Maleylacetoacetate is converted to its isomeric form fumarylacetoacetate by the enzyme maleylacetoacetate isomerase.
• Fumarylacetoacetate is converted to fumarate and acetoacetate in the presence of fumarylacetoacetate hydrolase (FAH).

**Figure 34.1 Tyrosine catabolic pathway:**

The schematic representation below shows tyrosine catabolic pathway and its related diseases. Enzymes involved in each step are listed (right) opposite to the disorders (left) caused by their deficiency. As shown, NTBC (2-[2-nitro-4-(trifluoromethyl) benzoyl] cyclohexane-1,3-dione or nitisinone is an inhibitor for hydroxyphenylpyruvate dioxygenase.
34.3 Types of Tyrosinemia

Based on the shortage of the enzyme (due to defective gene involved) there are three types of tyrosinemia.

34.3.1 Type I Tyrosinemia: It is the most severe form of tyrosinemia and is caused by a shortage of the enzyme fumarylacetoacetate hydrolase (FAH) due to defect in the FAH gene. Hereditary type I tyrosinemia affects 1 in every 100,000 children. Since they lack the enzyme FAH, so cannot convert fumarylacetoacetic acid into fumaric acid. Instead, fumarylacetoacetic acid is converted to succinylacetoacetate and then to succinylacetone which is toxic for liver, kidneys and brain.

Successful treatment is available nowadays with medication and controlled diet. The symptom of this disorder usually appears in the first few months of life and includes:

- Failure to thrive or failure to grow and gain weight at the expected normal rate.
- Jaundice, characterized by the yellowing of the skin and whites of the eyes.
- Cabbage-like odor
- Vomiting.
- Diarrhea.
- An increased tendency to bleed (particularly nosebleeds).

Type I tyrosinemia can lead to permanent liver and kidney failure. The nervous system is affected and increased chances of liver cancer persist in this disorder.

34.3.2 Type II Tyrosinemia: It is caused by a deficiency of the enzyme tyrosine aminotransferase (TAT) due to defect in the TAT gene. The organs affected by Type II tyrosinemia include eyes and skin. Impaired mental development has also been observed in patients with this disorder. The symptom of this disorder usually appears in the early childhood and includes:

- Excessive tear formation.
- Photophobia or abnormal sensitivity to light.
- Redness and pain in the eyes.
• Development of painful skin lesions on the palms and soles.
• Mental disability (about 50 percent of individuals affected with type II tyrosinemia have some degree of intellectual disability).

34.3.3 Type III Tyrosinemia: It is a rare disorder caused by a deficiency of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPD) due to defect in the HPD gene. Characteristic features of this disorder usually include:

• Intellectual disability
• Intermittent ataxia or seizures, and periodic loss of balance and coordination.

34.4 Treatments for Tyrosinemia

There are three strategies other than the gene therapy being used to treat tyrosinemia.

• Controlled diet (the use of a diet low in tyrosine and phenylalanine to minimize the amount of tyrosine that needs to be metabolized): A diet with very low phenylalanine and tyrosine should be started immediately upon diagnosis of tyrosinemia. The diet should support an appropriate rate of growth, normal intellectual development, provide adequate nourishment and prevent neurological crisis, liver and kidney dysfunction, and formation of tyrosine crystals in the eyes.
• Medication: Nitisinone or NTBC (brand name Orfadin®) is a medication that has shown good results.
• Liver transplantation: In case of acute liver damage there is no option but liver transplantation.

Determination of the efficacy of the treatment is monitored by frequently measuring the concentration of phenylalanine, tyrosine, succinylacetone, all amino acids, albumin, prealbumin, and haemoglobin in blood. Liver function studies should also be conducted.
34.5 Gene therapy for Tyrosinemia:

Several gene therapy strategies have been designed and are being clinically tested to overcome this disorder. Some animal trials have been discussed below. Sleeping beauty (SB) transposon mediated gene transfer, Adeno-associated virus mediated gene repair are some of the promising DNA delivery techniques of gene therapy for tyrosinemia.


FAH-deficient mice were used as a model in this study. By analyzing the expression and integration of FAH transposon in FAH-deficient mice they have tried to study the activity of the SB transposon in vivo. The clonal selection of stably corrected hepatocytes for FAH protein was done by different molecular techniques.

It was observed that:

- The SB-transposon-transfected hepatocytes resulted in long lasting correction of the FAH-deficient phenotype.
- Stable expression of FAH was obtained in transfected hepatocytes.
- The expression of the FAH was stable.

2. In another study Pan XJ et al., have explored the reliability of sleeping beauty transposon system as a gene delivery tool for hereditary tyrosinemia type I disease gene therapy. (Sleeping Beauty transposon system is a reliable gene delivery tool for hereditary tyrosinemia type 1 disease gene therapy: size of the foreign gene decides the timing of stable integration into the host chromosomes. Pan XJ, Ma ZZ, Zhang QJ, Fan L, Li QH. J Int Med Res. 2012;40(5):1850-9.)

Objective of the study: Using SB transposon system for correcting the loss of FAH in a knockout mice model.
**Sleeping Beauty transposon system used in this study:**

Plasmid maps of the Sleeping Beauty transposon system constructs used in this study are shown below.

A. The fumarylacetoacetate hydrolase (*Fah*)-SBTS construct contained the mouse *Fah* gene

B. The forkhead box M1b (*FOXM1B*)-Fah-SBTS contained the human *FOXM1B* gene conjugated with the mouse *Fah* gene using the internal ribosome entry site (IRES) sequence

C. The Luc-SBTS construct contained the firefly luciferase gene

**Method:** Twenty FAH knockout mice were used in the study with appropriate control animals.

- All FAH knockout mice were given NTBC.
- Firefly luciferase was used as a marker.
- Treatment of NTBC was stopped following injection.
- FAH knockout negative controls were kept healthy with continued NTBC administration.
- Body weight of Mice were recorded daily.
- *In vivo* bioluminescence help in evaluating of transgene expression.

**Results:** The FAH gene was integrated into the host chromosomes within a week of gene therapy.
Conclusion: The truncated FAH gene integrated easily and faster into the mouse chromosomes than the complete version and hence advantageous for stable gene therapy in mice model.


The study showed that the adeno-associated virus (AAV) vectors are ideal for performing gene repair. AAV vector has the ability to target multiple different genomic loci with lower immunogenicity. It is capable of targeted stable expression through integration. It is low mutagenic and a vector of choice for gene repair because its single stranded nature facilitates correction by homologous recombination. However, many drawbacks to gene repair therapy remain. Most notable drawback of this system is the low frequency of correction in vivo and thus is of no therapeutic value for any disease.

Method: A mouse model of hereditary tyrosinemia type I with a point mutation was used to test whether targeted AAV integration by homologous recombination could achieve high level stable gene repair in vivo or not.

To conduct this study both the neonatal and adult mice were treated with AAV serotypes 2 and 8 carrying a wild-type genomic sequence for repairing the mutated FAH gene.

Observation:

- Gene repair was successful with both serotypes having AAV-8 more efficient than AAV-2.
- Frequencies of correction were up to $10^{-3}$.
- It was observed that the repaired hepatocytes had a selective growth advantage and were able to proliferate efficiently. The repopulation of the mutant livers with corrected gene carrying hepatocytes occurred and the underlying metabolic disease was cured.
**Conclusion:** This study concludes that the AAV-mediated gene repair is feasible *in vivo* in mice model.