Chapter 2. Literature review

I. HYPOTHYROIDISM

A. Hypothyroidism- Types, Prevalence and Diagnosis

Most thyroid related disorders are chronic and are predominant in women than in men. Currently prevalence of thyroid related disorders are on rise. Thyroid is butterfly shaped small endocrine gland present in neck region and secretes thyroid hormones, thyroxin (T4) and triiodothyronine (T3) that are responsible for regulation of protein, fat and carbohydrate metabolism and have effect on each and every organ of body by way of affecting energy utilization. These hormones also have role in brain development, cardiac ventilation and endometriosis. Blood levels of T4 and T3 are regulated by levels of thyrotropin (thyroid stimulating hormone, TSH). An important component utilized in thyroid hormone synthesis along with amino acid tyrosine is iodine (1).

Hypothyroidism is an endocrine disorder characterized by decreased activity of thyroid gland leading to insufficient production of thyroid hormones. Subclinical or asymptomatic hypothyroidism is characterized by elevated thyrotropin level and normal serum thyroid hormones level. Whereas, there remains elevated TSH but decreased thyroid hormones serum levels in case of overt or clinical hypothyroidism (2) (3). Diagnosis of hypothyroidism is usually based on symptoms and results of blood levels of TSH, T3 and T4 and supported by plasma anti-TPO antibody estimation (4).

In India, hypothyroidism used to usually be categorized under the iodine deficient disorders and represented based on total goiter rate. Government of India has adopted the universal salt iodization program and since then there has been a decline in goiter prevalence in various parts of the country (5-9). As per World health organization (WHO) assessment report India has undergone transition from iodine deficient state to iodine sufficient state (10-12). A large, cross-sectional, comprehensive study recently carried out in adult population across the country, indicates about 10.9% prevalence of hypothyroidism (13); whereas, the prevalence of hypothyroidism in the developed countries is about 4-5% (14, 15). This indicates even though most of the regions of India have been made iodine sufficient there is still high prevalence of hypothyroidism. Hence, underlying pathogenesis may involve a complex interplay of genetic, environmental and endogenous factors and not only iodine deficiency. Clinical investigation of patients in India does not include evaluation of thyroid

autoantibodies and hence iodine deficiency is believed to be the sole candidate for hypothyroidism pathogenesis which may not be the case.

Autoimmune hypothyroidism is characterized by gradual destruction of the thyroid gland due to loss of thyroid cells, leading to thyroid hormone deficiency. The immunological features of this disorder include the presence of anti-thyroidperoxidase (anti-TPO) antibodies and, less commonly, anti-thyroglobulin (anti-TG) antibodies, abnormalities in the circulating T cell population and a goiter with lymphocytic infiltration (16, 17). Thyroid peroxidase (TPO) is a heme containing microsomal membrane glycoprotein (molecular weight- 105KDa) that catalyzes iodination and coupling of tyrosine residues for production of thyroid hormones in the thyroid gland (18). It is also a major thyroid autoantigen that elicits the production of autoantibodies found in the serum of many patients with autoimmune thyroid disease as well as other autoimmune diseases (19). Inhibition of TPO due to persistent autoantibody (anti-TPO) leads to decreased synthesis of thyroid hormones leading to autoimmune type of hypothyroidism also known as hashimoto's thyroiditis. Presence of anti-TPO antibodies in blood circulation has been used for clinical diagnosis of this disease in USA (20), Europe and many other countries(21) but not in India (22). Hypothyroidism diagnosis is limited to determination of thyroid hormone and thyrotropin serum levels and evaluation of autoimmune antibodies in patients is not currently practiced in India.

B. Pathogenesis of autoimmune hypothyroidism

Pathogenesis of autoimmune hypothyroidism involves complex interplay of several genetic and environmental abnormalities in multistep process. Disease initiation occurs with accumulation of MHC class II antigen presenting cells (APC) and macrophages in to thyroid due to any of environment triggering factors such as viral infection, iodine and toxins (23).

After accumulation in thyroid, APC present thyroid specific autoantigen to naïve T cells which leads to clonal expansion followed by maturation in to autoreactive T and B-lymphocytes in lymph node leading to production of auto-antibodies against thyroid autoantigens also occurs. What Triggers accumulation of APC and presentation of self protein as antigen to T cells is not clearly known but it is believed to occur due to loss of self tolerance as a result of complex interplay of genetic predisposition and environmental triggers. Finally, helper T lymphocytes mediate

attack of cytotoxic T lymphocytes and B lymphocytes on thyroid cells by inflammatory cytokines such as interleukin-12, interferon- γ and tumor necrosis factor-α. This substantial attack on thyroid causes destruction of thyroid follicles, where thyroid hormones are synthesized and stored in the form of thyroglobulin, leading to autoimmune hypothyroidism (23).

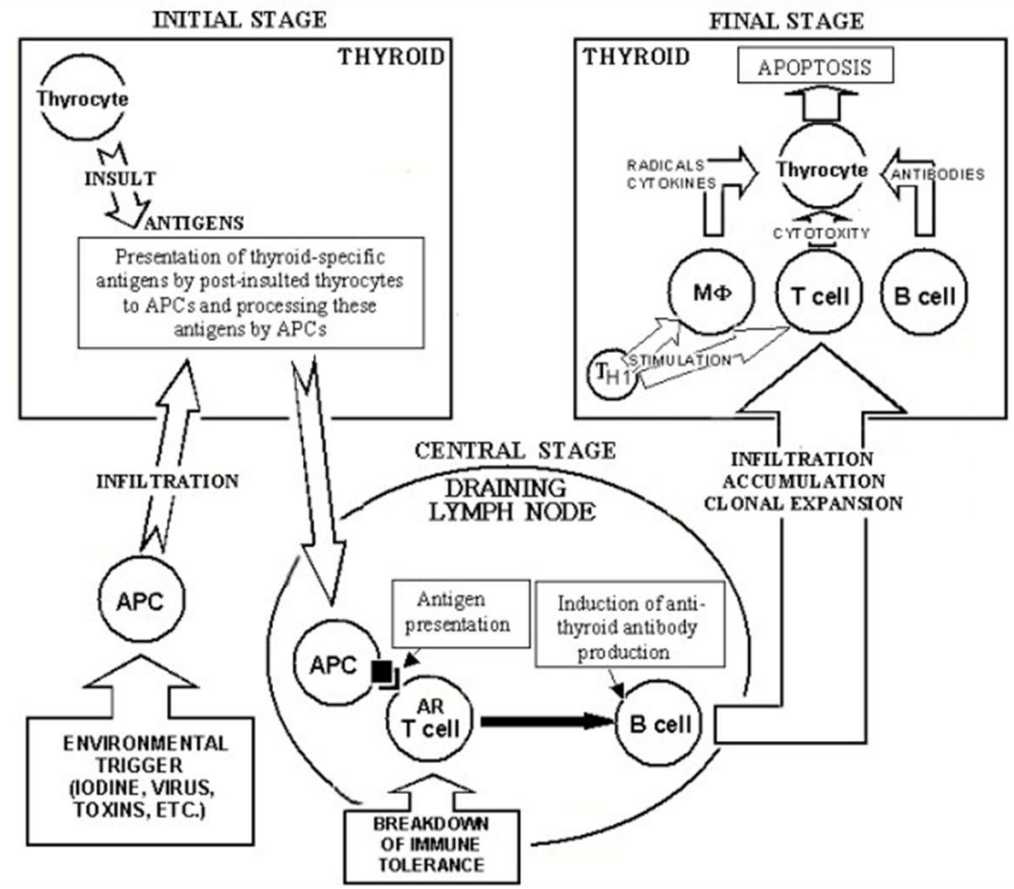

Figure 2.1 A schematic representation of autoimmune events that occurs in autoimmune hypothyroidism/ Hashimoto's thyroiditis. Adapted from "Immunogenetics of Hashimoto's thyroiditis" by Dimitry A et al **(24).**

C. Treatment and Management of hypothyroidism

All types of hypothyroidism are treated with synthetic thyroxine, a drug that is identical to thyroid hormone thyroxin. The correct dose for treatment is determined based on results of diagnosis tests and also depends on age and weight of patient, severity of the hypothyroidism, the presence of other diseases, and other ongoing medication. Plasma TSH levels are measured every 6 to 8 weeks after therapy to adjust dose for further treatment. After each dose adjustment thyroid hormone profiling is done. Process is repeated until stable dose is reached (4) (20). However,

supplementing thyroxine for treatment of hypothyroidism is symptomatic cure for autoimmune type of hypothyroidism. Actual treatment of autoimmune hypothyroidism should be capable of preventing gradual thyroid gland destruction and loss, a root cause of the disease.

D. Gene therapy for autoimmune hypothyroidism- Current Perspective

As presented in Figure 2.1, autoimmune hypothyroidism occurs due to complex interaction between genetic predisposition and an environmental trigger (dietary iodine, infection, pregnancy, cytokine therapy) (24). Identification of those gene that predispose an individual to autoimmune hypothyroidism can help in detecting risk of developing autoimmune hypothyroidism in particular individual. Furthermore, genes that are involved in pathogenesis of disease can be useful to identify new therapeutic targets and hence new therapeutic options for this disease.

Therapeutic potential of interleukin-10 (IL-10) has been proved in experimental autoimmune thyroiditis (EAT) mice model by *in vivo* administration of plasmid encoding IL-10. The direct injection of IL-10 pDNA expression was found to inhibit lymphocytic infiltration in thyroid and also alleviated the progression of autoimmune thyroiditis (25). Similarly, *in vivo* administration of Fas Ligand gene in experimental autoimmune thyroiditis has lead to destruction of autoreactive infiltrating T lymphocytes and reversal of EAT, indicating its therapeutic potential in management of autoimmune hypothyroidism (26).

II. GENE DELIVERY VECTORS

Delivery of exogenous gene in to target eukaryotic cell has been extensively studied using viral and non-viral vectors. Various viral vectors such as adenovirus, adeno-associated virus and retrovirus vectors have very high transfection efficiency but suffer from various drawbacks such as generation of immune or toxic reactions and insertional mutagenesis within host (27). Research in this field is currently driven by quest for safe and effective alternative of viral vectors for gene delivery. Non viral vectors for gene delivery include polymer and lipid based vectors. Complex of plasmid DNA (pDNA) with lipid is known as Lipoplex and polymer is known as Polyplex. These vectors provide safe alternative of viral vectors, higher flexibility and ease of manufacturing. Gene being negatively charged in nature, generally, cationic lipids and cationic polymers are used as gene delivery vector to form complexes. The

use of cationic lipid for gene delivery was first put forward by Felgner and termed the process of transfection of pDNA using cationic lipid as Lipofection (28). Ever since then cationic lipids are widely studied for gene delivery. They are commercially available as easy to use transfection agents such Lipofectamine and are even taken to clinical trials (29). The most widely used monovalent cationic lipid includes DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane) and DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate). Most widely used multivalent lipids include DOSPA ((+)-N,N-dimethyl-N-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride) and DOGS (dioctadecylamidoglycylspermine) (30). DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) is used as helper lipid in combination with these cationic lipids. Lipoplexes too have critical disadvantages related to stability upon storage and *in vivo* administration, reproducible formulation and toxicity. Polymers used for gene therapy are of various types, linear, branched and dendritic, among all most widely studies are Polyethyleneimine (PEI) (31), Poly L-lysine (PLL) (32) and Polyamidoamide (PAMAM) (33). These polymers provide stable polyplexes, have flexibility for modification of functional groups such as primary amino groups, and provide ease of manufacturing. However, poor transfection efficiency and toxicity have limited their clinical utility.

III. BARRIERS AND STRATEGIES TO IMPROVE TRANSFECTION EFFICIENCY

To be effective gene delivery vector, cationic lipid or polymer should be capable of delivering gene to cell nucleus by successfully crossing various extracellular and intracellular barriers. Detailed understanding of these barriers can help to device strategies that can help to overcome such barriers (34).

A. Extracellular barriers

First barrier that any gene delivery vector faces before entering cell is extracellular barrier. These barriers include stability of vector in serum after delivery in to blood stream. An effective non-viral gene delivery vector should be able to maintain its complex with pDNA so as to protect pDNA from extracellular nucleases so that it can be transported through blood vessels and surrounding tissues to the cellular level. Stability of non-viral gene delivery vector in serum is affected by physiological salts

and plasma proteins such as albumin which causes displacement of pDNA and makes it vulnerable to degradation by serum nucleases. Therefore non-viral gene delivery vector should be able to withstand such challenge.

Polyplexes or any other vector for gene delivery should be able to block access of such enzyme to pDNA. Unprotected pDNA are degraded within few minutes in serum. Whereas, polyplexes have ability to protect them for hours if pDNA is condensed in to small compact particles of nanometer size range by electrostatic interaction between negatively charged phosphate groups in pDNA and positively charged function groups (generally amine groups) in cationic polymer DNA. Complex formation and condensation occur instantaneously due to interaction between pDNA and cationic polymer and leads to formation of spherical or toroid shape particles (33, 35, 36).

Each polyplex most often comprises several pDNA molecules along with many polymer chains. The structure and morphology of polyplexes seems to be kinetically controlled and often depends on the order of mixing (for example, adding polymer to DNA solution or DNA to polymer solution). Efforts have been made to better understand polyplex formation but improvements in the theoretical understanding of the process and physicochemical characterization of the resulting complexes are needed (35, 37, 38). Structure of cationic polymer i.e. linear or branched or dendrimer and degree of branching also affect condensation and pDNA binding. Minimum six to eight cationic charge is required, for peptide mediated gene delivery, for efficient pDNA condensation (39-41).

Some group has shown that substitution of polycationic polymer such as PLL using amino acid can increase binding of PLL with pDNA. However, other group has found the opposite effect in terms of decrease in complexation potency of branched polycation. This difference may have been observed due to different types of polymer used in these two different studies. However, increase in binding does not correlate with increase in transfection efficiency. In some cases tight binding may also lead to decrease in transfection efficiency. In conclusion, balance between binding capacity required to protect pDNA in serum and ability to release pDNA to show transfection efficiency is essential (41, 42).

1. Serum stability

The stability of polyplexes depends on charge of polyplexes. Due to positive charge on surface polyplexes may interact with plasma proteins and form aggregates. These aggregates are rapidly cleared by reticuloendothelial system. Modification of polyplex surfaces to conceal positive charge or hydrophobic nature of surfaces, various hydrophilic moieties such as Polyethylene Glycols (PEG) and sugars are attached which can help increase circulating half life of polyplexes (43). Increased stability due to such modifications of surface results because of stearic hindrance provided by their long chain, degree of surface modification and method used. Surface modification increases serum stability but at the cost of transfection efficiency. Hence optimization of surface modification by its effect on transfection efficiency and serum stability is needed (43).

B. Intracellular barriers

After successfully crossing all extracellular barriers gene delivery vector face intracellular barriers. Gene delivery non-viral vector enter cells by endocyctosis and hence endocytic pathway can lead them to lysosomes where they are finally digested in acidic environment by degradative enzymes. Non-viral vector must escape this pathway and enter cytoplasm and lead pDNA to nucleus as well as release pDNA at same time so that further transcription and translation can occur (44) (45).

1. Endo-lysosomal barrier

Gene delivery vectors are internalized in cell by endocytosis pathway and end up to the hostile environment of endosome. These endosome can fuse with early endosome which again fuse with cell membrane and transported back out of the cell. However, gene delivery vectors do not fuse with early endosome but with late endosome. Late endosome have ATPase proton pump enzyme in the vesicle membrane which can acidify endosome vesicle to pH 5-6. These late stage endosome empty its content to lysosome which has pH ~4.5 and also contain various enzymes. At this stage gene delivery vector along with pDNA are degraded. Only few pDNA or gene delivery vectors that escape this degradation have chance to reach cell nucleus.

Various strategies have been used to overcome this barrier. First method tried for endosomal escape was to use chloroquine along with gene delivery vector. Chloroquine has capacity to buffer endosomal pH. This approach was tried for *in vitro* application. However, this is not practicable for *in vivo* application. Other

researchers have attached inactivated viral particles to delivery vector which showed man fold increase in transfection efficiency and results were attributed to endosomal escape provided by inactivated viral particles. However, these viral particles can also aid subsequent step of gene delivery such as nucleus entry. So overall increase in transfection efficiency may also be combined effect on all steps of gene delivery process. At the same time this inactive particle preparation, isolation and their fabrication on the surface of polymeric gene delivery vector surface is also challenging, consideration of this in connection with immunogenicity concerns and safety also makes it impracticable (46, 47). Alternatively, use of peptides or fusogenic viral particles attached to the surface of gene delivery vector has resulted in increased transfection efficiency (48, 49). Certain cationic polymers such as PEI (50) and fusogenic lipids such as DOPE (51) also have ability to escape from this barrier.

2. Transport through cytoplasm

Once gene delivery vector escape endosome it has to travel in cytoplasm to cell nucleus. Mobility of vector in cytoplasm is restricted by cell organelles and proteins and is directly dependent on size. After pDNA is released from vector in cytoplasm, size of pDNA affects its mobility in cytoplasm. Generally pDNA with size greater than 3000bp in length move very slowly in cytoplasm and makes it more vulnerable to degradation by cytosolic nucleases (52). Gene delivery vectors being negatively charged in nature can move towards due to microtubules during mitosis. However, mechanism of their transport to nucleus needs to be studied in detail in order to improve design (53).

3. Nuclear localization

Nucleus localisation is required so that pDNA can utilize transcription machinery of nucleus and further in form of mRNA translate in to protein. Most important barrier for nuclear localization is nuclear double bilayer membrane with tightly regulated pores that allow only specific set of biomaterials to enter inside nucleus. For viral vector this is not a major barrier but for non-viral vector nucleus membrane is major barrier for transfection. Non-viral vectors cannot evade nuclear membrane and has to wait for cell division during which nucleus membrane breaks. Hence, cells that are transfected immediately before cell division show greater transfection efficiency (54, 55). Nuclear localization mechanism is not well studied but much work has been done to understand nuclear localization signals (NLS). NLS are short cationic sequences

that are recognized and taken up by importins. Nucleotide sequences are also recognized and taken up inside the nucleus (56, 57). Use of cationic peptides that are substrate for NLS can help in nuclear localization of gene delivery vectors.

4. Unpackaging

At some point of time after endosomal escape polyplexes should be able to de-complex to set free pDNA by way of unpackaging. Polyplex by way of complexation prevent pDNA from degradation *in vivo* but at the same time prevent pDNA from translation and hence unpackaging is essential. Various lipophilic modification, conjugation with polyethylene glycol and use of low molecular weight PEI help in this process (58).

IV. POLYETHYELENEIMINE AS GENE DELIVERY VECTOR

PEI is widely explored polymer for gene delivery ever since its discovery (31). Due to presence of primary amino groups in its structure it is easy to modify it for targeting. It has been used to target specific cell type using transferrin (59), mannose (60), galactose (61) and antibodies (62). Various studies have proved that it is capable of transfecting of variety of tissues such as kidney, lung, central nervous system and tumors. However its relatively high toxicity has limited or hindered its use. At present it is believed that cationic nanomaterials are internalized majorly through clathrin-mediated endocyctosis and to lower extent by macropinocytosis. However PEI based polyplexes are internalized using multiple pathways such as caveolae-mediated endocyctosis in addition to clathrin-mediated endocyctosis and macropinocytosis (63).

After internalization due to any of the routes mentioned above, further transfection efficiency of PEI is believed to be due to presence of protonable nitrogen atoms in amino groups of PEI. Because of considerable presence of nitrogen atoms in PEI it has buffer capacity in acidic environment of lyososomes (64). This mechanism was described by Behr et al in 1997 as a trick to enter cells (50). Acidic environment of lysosome is responsible for accumulation of weak bases in to these vesicles which after protonation cannot diffuse back in to cytosol (65). Agents that accumulate in lysosome are known as lysosomotropic agents and then they get trapped inside lysosome at hundred fold high concentration than cytosol (66). Many pharmaceutical drugs namely chloroquine, chlorpromazine, imipramine, have such lysosomotropic

effect. However, this effect may lead to cytotoxicity. In case of PEI, once inside lysosome, it absorbs protons resulting in more protons being pumped by V-ATPase pump inside lysosome to maintain its acidic pH. This proton influx by ATP dependent pump also leads to simultaneous chloride ion and water influx. These events further lead to ostmotic swelling of lysosome leading to rupture of lysosome and subsequent release of its content in to cytoplasm (50). Many researchers have tried to confirm proton sponge effect by employing different strategies and have confirmed it. However, few researchers believe that proton sponge effect to be incorrect because in their experiment they failed to observe PEI mediated increase in pH of lysosome (67). In order to confirm results obtained in their study, when other researchers performed same study, they found that dye which showed no increase in pH of lysosome does not co-localize with PEI and hence cannot truly confirm Proton sponge hypothesis (68). Few researchers believe that proton sponge effect is not the dominant mechanism for PEI and other mechanisms may also exist such as escape of few PEI polyplexes from endosome before they reach lysosome (69). It is therefore reasonable to assume that increase in protons inside lysosome occurs and hence proton sponge hypothesis is correct but no change in pH of lysosome occur. However, proton sponge hypothesis is widely accepted mechanism for PEI based transfection. Mechanism of nuclear entry of PEI is not well understood but it may occur due to interaction of negatively charged nucleus membrane or translation may occur during mitosis without need to cross nucleus membrane. However, even if mechanism of nucleus entry is not know, it has been reported that PEI alone or in complex with pDNA undergo nuclear localization in form of ordered structure (70).

V. POLYETHYLENEIMINE MEDIATED CYTOTOXICITY

Linear as well as branched PEIs are cytotoxic in nature and their mechanism of cytotoxicity is well studied (71). PEI induces its toxicity in two ways. First, it induces necrosis like changes in plasma membrane within 30 min of exposure leading to compromised plasma membrane integrity and membrane perturbation. Changes in plasma membrane include translocation of phosphatidylserine from inner plasma membrane to out surface. Second, PEI induces channel formation in mitochondrial membrane which leads to release of proapoptotic cytotchrome-C in cytosol. This is followed by caspase-3 translocation in to mitochondira which leads to activation of caspase-3, which is crucial mediator of programmed cell death, and disturbance in

mitochondrial potential. These leads to mitochondria mediated programmed cell death i.e. apoptosis which occurs ~24hr after PEI treatment. In conclusion, PEI induces early necrosis as well as late apoptosis based cytotoxicity.

VI. LOW MOLECULAR WEIGHT PEI AND ITS MODIFICATIONS

Transfection efficiency of PEI is dependent on its molecular weight and polydispersivity. High molecular weight (HMW) PEIs are more efficient as transfection agents than low molecular weight (LMW) PEI. At the same time they also show high cytotoxicity in comparison to LMW PEIs (72). Various modifications of LMW PEIs have been performed till date to improve its transfection efficiency and of HMW PEIs to reduce its cytotoxicity.

LMW PEIs are modified using endosome disruptive agent lysine-histidine peptide conjugated to 6-bromo hexanoic acid using ethylene diamine as a linker (73), poly(ε-caprolactone) to form diblock copolymer (74), lipoic acid to form reduction triggered conjugate (75), and other hydrophobic modification of LMW PEIs performed using Carboxylic acid NHS ester (76) and folate NHS ester (77), 1-iodo dodecane and 1-iodo hexadecane (78), fatty acid chlorides (79-81). Pluronic (82-84), PPO-PEO (polypropylene oxide- polyethylene oxide) (85), poly-γ-benzyl-L-Gluatamate (86) and, Cholesteryl chloroformate (87-92). Low molecular weight PEI can be utilized to overcome their inherent limitations by suitable chemical modification to make them effective gene delivery vectors.

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