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CURRENT TRENDS IN THE GENE THERAPY OF HEMATOLOGIC DISORDERS

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Abstract. *Recent advances in molecular genetics and the invention of new technologies led to an advance in the development of gene therapy. Gene therapy is used to correct defective genes in order to cure a disease or help the body better fight a disease. It works by restoring or modifying cellular functions through the introduction of a functional gene into the target cell. The concept of gene therapy is simple, but introducing it to routine clinical practice is not. The main concerns are related to some safety issues as well as to the problem that maintaining a stable and prolonged expression in target cells may not be easily achieved. In spite of the difficulties, gene therapy remains a hope for many hematological disorders that cannot be effectively treated so far. This article reviews the current status of gene therapy with a focus on hematological disorders. In addition, clinically applied approaches are presented through particular examples of approved gene therapy drugs.*

Key words: *gene therapy, hematological diseases, gene therapy drugs*

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Advances in gene therapy and molecular techniques have led to the rapid development of safer and better tolerated therapies for many patients with hematologic cancers. Clinically functional "gene therapies" are established now for certain diseases, and many other promising therapeutics have been developed, mostly noticeable in the field of hematological diseases. It is one of the main fields where many novel concepts, approaches, and technologies are applied first. The reason behind such a trend may be the easiness of isolation of individual cells. For hereditary diseases in hematology, the advance in delivery system (particularly, the advance of the viral vector system) has contributed greatly to the improved clinical outcomes with reduced genotoxicity. Such a trend is most evident in ex vivo gene therapy of hereditary diseases. Another recent advance has been the application of gene editing technologies for the cure of human diseases. Zinc finger nucleases, as well as Cas/CRISPRs and TALENs can be used to edit target genes, achieving knock down or repair. These technologies have great potential to clinically "destroy" or "repair" the mutant gene back to normal without causing genotoxicity in other genes.

On the other hand, the correction of 100% of malignant cells is difficult to achieve in actively proliferating cells and the normalization of the initial inducer

mutation does not necessarily kill all malignant cells. Therefore, some treatments were designed for elimination of malignant cells. Recent advance of CAR-T cell therapy, including its application to B cell malignancies, is a lead example of such an approach. Immuno-gene therapy (such as GVAX, GM-CSF producing whole tumor cell vaccine) has shown promising results, RNA interference and suicide gene-based regulation of GVHD have been actively tested for hematological malignancies.

This review article aims to summarize recent advances in the gene therapy of hematological diseases and to give actual examples of approved drugs based on the discussed approaches.

CAR-T TREATMENT

Second-generation chimeric antigen receptors (CARs) are powerful tools to redirect antigen-specific T cells independently of HLA-restriction [1]. CARs are hybrid receptors comprising a ligand for a cell-surface molecule, most often consisting of a single-chain variable fragment (scFv) derived from a monoclonal antibody or an antigen-binding fragment (Fab) fused to signaling domains assembled to redirect T-cell function [2]. The targeted antigen could be a protein, a carbohydrate or a glycolipid. CAR T cells therefore express two receptors for antigen: their endogenous TCR and the transduced CAR [3]. CAR T cell production for clinical purposes begins with collection of T cells from the patient, which is accomplished by leukapheresis. T cells are isolated and activated by antibodies specific for CD3 and/or CD28, which induces T cell activation and proliferation. The transduced cells are expanded until the required CAR T cell dose is achieved. CAR T cells then undergo pre-defined quality control and assurance assays to confirm function and sterility before the product is released for clinical use [4].

Clinical studies evaluating CD19-targeted T cells in patients with B-cell malignancies demonstrate the potency of CAR-engineered T cells. The parameters which best determine T-cell function and clinical outcome of CAR-based approaches are CAR design, T-cell production methods, conditioning chemotherapy as well as patient selection [5].

First-generation CARs presented with limited T-cell activation, cytotoxicity but only short-term T-cell expansion. Second-generation CARs, which combine activating and co-stimulatory signaling domains, enable improved cytokine secretion, T-cell expansion and persistence [6]. CARs have been generated against several cell surface molecules, like CD19, HER2, GD2, prostate-specific membrane antigen (PSMA) and mesothelin. The most promising clinical outcomes of this technology have been reported in patients treated with autologous CAR-modified T cells targeting CD19 [7, 8]. CD19 is expressed by most B-cell leukemias and lymphomas but not in tissues of normal B lineage cells [9]. Common toxicities of patients with chronic lymphocytic leukemia (CLL) undergoing infusion of autologous CD19-targeted T cells well, included fever, hypotension, lymphopenia and delayed tumor lysis syndrome [7, 8, 10, 11]. No deaths directly attributed to the infusion of CD19-targeted T cells have been reported.

Davila et al. [5] reported an inverse correlation between a detectable persistence of CAR-modified T cells and disease burden at the time of T-cell infusion. CLL tumor burden is estimated by taking into account circulating tumor cell counts, the amount of tumor cells in the bone marrow and peripheral tumor masses [12]. Calculations made the additional assumption that 1×10^{12} CLL tumor cells held an equivalent weight of 1 Kg. Utilizing this tumor burden calculation, the authors concluded that patient tumor loads in the bone marrow and blood ranged from 8.8×10^{11} to 3.5×10^{12} CLL tumor cells. The degree of treatment response in this small sample size was inversely proportional to tumor burden, with the patient bearing the lowest tumor mass achieving the best clinical response.

Another parameter, influencing the successfulness of CAR-T treatment is the pre-infusion conditioning chemotherapy. The best results were achieved by conditioning chemotherapy that probably resulted in lymphodepletion [8, 13]. However, in contrast to chemotherapeutic agents, infused modified T cells may undergo significant expansion under optimal conditions, as well as rapid disappearance under suboptimal conditions. Cumulated data suggest that there is no correlation between T-cell dose and clinical response [5].

An interesting advance to increase safety and/or efficacy of this approach is the creation of *bi-specific* CARs. T cells were genetically modified to express 2 fully autonomous CARs (CAR1 and CAR2) specific for 2 distinct tumor antigens [14, 15]. A different principle was used to limit off tumor activity, by co-expressing a CAR and a CCR [14, 15]. In this case, ligation of the CAR or CCR by its respective antigen alone is insufficient to induce full T cell activation. However, their ligated leads to full T-cell activation.

SUICIDE GENE THERAPY FOR GVHD

The basic strategy of this approach is to genetically modify donor T cells ex vivo before infusion for pre-

vention of viral infections. Different methodologies have been evolved to introduce a suicide gene into the T lymphocytes before infusion into patients. A number of genetic systems have been used or explored experimentally [16]. Two have been tested in clinical trials, one with a vector having the thymidine kinase gene, and post-treatment is with ganciclovir [16]. The other system involves caspase-9 [17]. T cells containing this gene can be abrogated using a dimerizing drug (AP1903). Overall, this approach shows promise for control of GVHD.

RNA INTERFERENCE AND GENE SILENCING

Double-stranded RNA (dsRNA) is the most potent and preferred way to target specific genes [18]. Endogenous regulatory microRNAs (miRNA) measure ~22 nucleotides and are generated from processing of long primary transcripts (pri-miRNAs) into stem-loop precursors of ~70 nucleotides. Exogenous delivery of short interfering RNA (siRNA) bypasses the need for transduction and nuclear processing, but rapid degradation by ribonucleases limits systemic therapy applications. Immunomodulatory approaches with RNAi have been studied in several cancers and inflammatory conditions, such as rheumatoid arthritis, in which silencing of TNF, IL1, IL6, and IL18 improves pathologic changes associated with the disease [19]. Recently, RNAi mediated silencing of the MLL fusion protein (MLL-AF9) in precursor B cell ALL silenced the leukemogenic fusion gene causing maturation arrest and ceasing the malignant behavior of these cells [20].

USAGE OF HSC – AUTOLOGOUS AND ALLOGENIC STEM CELL TRANSPLANTATION

HSCs are multipotent stem cells, generally defined by their expression of the CD34 antigen, and mainly reside in the bone marrow. They constitute less than 0.1% of cells in peripheral circulation, and have traditionally been harvested directly from the bone marrow via the iliac crest. As an alternative, HSCs can be harvested from the peripheral blood following treatment with hematopoietic growth factors, such as granulocyte-colony-stimulating factor (G-CSF; also known as neupogen or filgrastim). In a typical HSC collection from peripheral blood, donors receive G-CSF twice daily over 4-5 days, followed by harvesting via leukapheresis. A leukapheresis unit contains all the white blood cells, which have been separated from plasma and red blood cells based on density centrifugation. In addition to the CD34+ HSC population, the white blood cell compartment contains lymphocytes, granulocytes and monocytes. A patient collection yields over 6 million CD34+ cells per kg to ensure that a minimum number of 3-5 million gene modified cells per kg are finally re-introduced to the patient. This is often achieved in patients mobilized with G-CSF, but not in all cases, such as in those with sickle cell disease [21], where HSCs mobilize rather poorly with G-CSF, which results in an insufficient number of CD34+ cells being collected. More recently, plerixafor (an inhibitor of C-X-C chemokine receptor type 4 (CXCR4), a receptor responsible for the homing of HSCs to the bone marrow) has been used to either complement G-CSF mobilization or on its own, which has resulted in substantial increases in CD34+ stem cell harvests [22]. In spite of that, in some cases as Fanconi anemia, it is still difficult to obtain adequate numbers of CD34+ cells [23].

Hematopoietic stem cell transplantation (HSCT) is a globally accepted practice for the treatment of malignant and non-malignant disorders of the blood and immune systems, mainly leukemia, lymphoma and myeloma [24]. HSCs are relatively easy to acquire and transplant and possess both the capacity for self-renewal and the potential to differentiate to any hematological cell type. The HSCT is used to replenish the bone marrow and reconstitute the immune system with functional hematopoietic lineages. Allogeneic HSCT, i.e. transplantation of HSCs harvested from a healthy donor, is essentially the only option for cure of rare inherited diseases of the blood and immune systems. The limitations are the requirement of a genetically matched donor (which may not be available for up to 70% of cases), graft rejection, delayed immune reconstitution, graft-versus-host disease and a significant rate of mortality. Allogenic HSCT transplantation is also applicable when a gene modification correction is needed as primary immune deficiencies, hemoglobinopathies, lysosomal storage and metabolic diseases, and bone marrow failure syndromes [25]. The primary immune deficiencies are also treated with gene-modified HSCs [6]. These include ADA (adenosine deaminase deficiency) and X-linked SCID, Wiskott-Aldrich syndrome (WAS) and chronic granulomatosis disease (CGD). Autologous transplantation of the patient's own HSCs, which have been gene-modified to correct for the underlying genetic cause of the disease, would be the preferred form of treatment for these patients.

A breakthrough research in stem cell biology are the attempts to induce pluripotent stem cells (PSCs) from patients mature cells (induced PSCs (iPSCs)) [23]. These autologous stem cells are expandable and have the potential to differentiate into theoretically any mature cell type. Genetic modification of haematopoietic stem cells (HSCs) for therapeutic

applications has been allowed by the European Medicines Agency with the approval of two drugs. The first, Strimvelis (GlaxoSmithKline, UK), is indicated for treatment of a rare primary immune disorder, known as adenosine deaminase deficient severe combined immunodeficiency (ADA-SCID) [26]. Following transplantation of autologous gene-modified HSCs expressing functional ADA, a 100% survival over a median follow-up of seven years was shown for 18 study participants [27]. The second treatment, Zynteglo, Bluebird Bio, USA, is based on the replacement of functional beta-haemoglobin in HSCs from patients with transfusion-dependent beta-thalassaemia. Data from clinical development show that patients treated with Zynteglo achieved either complete transfusion independence or a near two-thirds reduction in the annual number and volume of blood transfusions [28].

Successful HSC gene therapy ultimately depends on the ability to engraft adequate numbers of gene-modified HSCs that will persist in the long term and express therapeutic genes in all cells of the blood lineages. The treatment procedure is however complex and various aspects require careful consideration, including: (i) the collection of high numbers of HSCs; (ii) safe and efficient cell processing; and (iii) optimal transplantation protocols.

VIRAL VECTORS DELIVERY

The idea of using retroviral vectors to directly insert genetic material into nuclear DNA was introduced In the 1980s [29]. The ease with which blood is isolated and can be manipulated makes hematopoietic cells particularly good candidates for gene therapy applications [30]. Viral vectors form the cornerstone of gene-modified cell therapies. They are derived from natural viruses, but have been reconstructed to infect and optimally deliver genetic material to target cells. Many varieties of viral vectors exist, some of which allow for integration of therapeutic genes (e.g. retroviral and lentiviral), while others deliver genetic material for a transient effect (e.g. adeno- and adeno-associated viral vectors).

- *Adenoviral and adeno-associated viral vectors (rAAVs)*

Vectors based on adenovirus have a large transgene capacity allowing large or multiple gene expression cassettes to be included within the vector genome [31, 32]. Adenoviral vectors have had limited use in the treatment of blood disorders, although they have been quite effective in facilitating the generation of viral-specific T-cell populations [33].

rAAVs are less immunogenic than adenoviral vectors [34, 35]. Only the inverted terminal repeats are required as part of the vector genome, and packaging can be achieved to develop high-titers of the vector. Further, the incidence of preexisting immunity is generally low, particularly for certain serotypes such as serotypes 8 and 9. However, rAAV can carry only a transgene up to 4700 nucleotides. The AAV genome is single stranded, and vector preparations are composed of a mixture of vector particles having 1 of the 2 strands of the virus. Upon transduction, the required annealing of the 2 strands delays gene expression [33].

- *Gamma-retroviral (γ-retroviral) and lentiviral vectors*

For integration of therapeutic genes, gamma-retroviral and lentiviral vectors are mainly used for gene modification of cells of the hematopoietic system. Following transduction of patient cells, the viral RNA is converted to DNA by reverse transcriptase and then integrated into the cellular genome in a semi-random fashion using the integrase enzyme provided by the viral vector. The primary concern for using viral vectors is insertional mutagenesis. Initial clinical studies showed that HSC gene therapy using gamma-retroviral vectors was curative for the majority of primary immune deficiency patients (with ADA-SCID, X-SCID, WAS and CGD), but they were later marred by the occurrence of treatment-related hematological malignancies. After 2 years of follow-up, 25% of the patients presented with myelodysplasia or leukemia due to insertional mutagenesis caused by the gammaretroviral vectors [25]. Essentially, gamma-retroviral vectors typically insert near proto-oncogenes, which resulted in their activation and malignant transformation of the gene-modified cells. Interestingly, with over 45 patients treated to date, no ADA-SCID patients receiving gene therapies with gamma-retroviral vectors have thus far developed malignancies. The reasons for this remain unclear.

On the other hand, lentiviral vectors are associated with low immunogenicity and have been shown to integrate more randomly into the genome without risk of insertion near proto-oncogenes. Notably, HIV belongs to the family of lentiviruses, and even with all the cases of HIV infection, there have been no reports of associated hematological malignancies. Lentiviral vectors are able to infect a broader range of target cells, including non-dividing cells. Even with lentiviral vectors, transduction rates can be as low as 10% in CD34+ cells compared with > 90% in T cells when using equivalent ratios of vector to target cells.

Attempts have been made with other viral types. Foamy viruses, another type of retrovirus, do not cause disease and have not infected humans. The genome of foamy viruses integrates diffusely in cellular DNA rather than within genes or at regulatory elements [36]. The α-retroviral vectors are being considered for therapeutic gene transfer as well [37]. Such vectors integrate more uniformly within the genome, particularly within intergenic regions [38]. The therapeutic potential for this class of vectors has been shown by functional correction of chronic granulomatous disease (CGD) in a cell line as well as within transduced human cells that engraft in the mouse model [37].

- *GVax approach*

HLA-negative CML cells have been engineered to express GM-CSF, mixed with irradiated patient derived CML cells. It can be given as an intradermal vaccine to maintain deep remission [39]. Combination of the GVAX (GM-CSF-producing whole tumor cell vaccine) approach with innate immune activation has also recently gained increasing attention [40]. The Toll-like receptors (TLRs) are evolutionarily conserved pattern recognition molecules capable of sensing pathogenic molecular motifs expressed on invading microorganisms [41]. Both natural (i.e. LPS, CpG DNA, dsRNA) and synthetically formulated TLR agonists induce differential gene expression programs that activate evolutionarily conserved immune effector mechanisms in neutrophils [42], mast cells [43], and NK cells [44]. The GVAX approach has also shown promising results in a study of 19 chronic myelogenous leukemia (CML) patients receiving imatinib mesylate (Gleevec) therapy in combination with a GM-CSF expressing autologous tumor cell vaccine. Complete molecular remissions and deep responses have been detected [39]. Further development and refinement of GVAX approaches are needed before the promise of in situ and whole cell tumor vaccination can be fully realized.

GENE EDITING AND TRANSPOSON-BASED SYSTEMS

In contrast to older methods used for genome modification these editing tools target specific sites in the human genome for modification using site-specific nucleases. There are four classes of these nucleases: engineered *meganuclease, zinc-finger nucleases (ZFNs)*, *transcription activator-like effector nucleases (TALENs)*, and the more recent *CRISPR-Cas9*. The four types of nucleases share the same overall mechanism in which the nuclease protein makes a double-strand break (DSB) in the targeted locus DNA. This DSB is naturally repaired by one of two methods: (1) non-homologous end joining (NHEJ) where the two DNA ends are joined with occasional deletion or insertion of few nucleotides or (2) homology-directed repair (HDR) where homologous DNA is used as a template to repair the DSB. While the first method can be used to disrupt a gene of interest, the second method can be used to introduce, delete, or modify one or more nucleotides if coupled with an exogenous piece of DNA homologous to the targeted genomic region [45]. The four techniques have been used in gene therapy studies. A major adverse effect associated with these tools, however, is the off-target effect (OTE) where the introduction to the target site could lead to deleterious outcomes, such as activation of oncogenes or inactivation of tumor suppressor genes. Another adverse effect associated with engineered nuclease is cytotoxicity resulting from sustained expression of these nucleases. In the context of HSC gene modification, gene-editing techniques are generally poorly efficient relative to viral vectors. They are still proving to be save enough before being clinically applicable.

Transposon-based systems make up the second class of non-viral gene-modification strategies. Transposons are naturally occurring and mobile DNA elements that have the ability to change their positions in the genome. Within their structure, they have a recognition domain for transposase – the enzyme performing the 'cut and paste' function. Following excision via transposase, the transposon is inserted in a semi-random manner in the genome of patient cells [25]. Three transposon-based systems have been developed for therapeutic purposes, namely Sleeping Beauty, PiggyBac and Tol2. Notably, a transposon-based system is being successfully applied for creating CAR T cells, with development being in clinical trial stages [46, 47].

GENE THERAPY EX VIVO

Instead of introducing genome-editing tools directly to patients, target cells can be genetically modified ex vivo then transferred back to patients. This avoids adverse immunogenic effects associated with foreign particles and provides an opportunity to select for modified cells and enrich them before delivering them back to patients.

The successful engraftment of HSCs depends on the "right" conditioning of the patients. The aim is to create a niche within the bone marrow where the transplanted HSCs are able to home and engraft. Conditioning regimens can be in the form of total body irradiation and/or cytotoxic/chemotherapeutic agents, and can be administered at high doses to achieve maximal

ablation (myeloablation) of the bone marrow. In the short term, patients suffer complications such as mucositis, neutropenia and a high risk of infection, while in the long-term risks of sterility, organ damage and secondary tumors. Given these safety concerns, several groups have started to develop alternative preconditioning regimens. Novel approaches include the use of immunotoxins and monoclonal antibodies directed to markers expressed on HSCs, such as c-Kit [48] c-Kit and CD47 [49] and CD45 [49, 50].

FDA APPROVED GENE THERAPIES FOR THE TREATMENT OF HEMATOLOGICAL DISEASES

- *Yescarta (Axicabtagene Ciloleucel)*(Kite Pharma), approved for the treatment of adult patients with relapsed or refractory large B-cell lymphoma after two or more lines of systemic therapy. The patient sits for leukapheresis, and the product is sent to a lab. There, the T cells are genetically enhanced to attack cancerous cells and expanded.
- *Kymriah (Tisagenlecleucel)*(Novartis Pharmaceuticals), approved for patients up to 25 years of age with B-cell precursor acute lymphoblastic leukemia (ALL) that is refractory or in second or later relapse. Patient's T cells are modified to recognize CD19 on B cells, expanded, and reintroduced to the patient to combat Large B-cell Lymphoma.
- *Zynteglo (Approved in EU)*(Bluebird Bio),approved for patients 12 years and older with transfusion-dependent β-thalassemia (TDT) who do not have a β0/β0 genotype, for whom hematopoietic stem cell (HSC) transplantation is appropriate but a human leukocyte antigen (HLA)-matched related HSC donor is not available. Hematopoietic Stem Cells (HSCs) are collected from the patient, then treated with a lentiviral vector to introduce functional genes for beta-globulin. HSCs are then re-introduced to the patient.
- *Tecartus (brexucabtagene autoleucel)*(Kite Pharma), approved for the treatment of adult patients with relapsed/refractory mantle cell lymphoma (MCL). Patient undergoes leukapheresis. Lymphocytes are expanded and transduced with genes that strengthen the immune response to CD19+ cells. Autologous anti-CD 19 CAR T cells are reintroduced to the patient intravenously.

GENE THERAPY IN PHASE 3 CLINICAL TRIALS

- *AMT-061* (Etranacogene Dezaparvovec)(uniQure), Hemophilia B

Indication: For the treatment of patients with severe and moderately severe hemophilia B. Methods: Delivery of factor IX gene via adeno-associated virus serotype 5 (AAV5).

- *OTL-103* (Ospedale San Raffaele – Telethon Institute for Gene Therapy (OSR-TIGET))

Indication: Wiskott Aldrich Syndrome Methods: Patient-derived Hematopoietic Stem Cells (HSCs) are transduced with functional copies of nonfunctional genes via a lentiviral vector.

- *BENEGENE-2 (Fidanacogene Elaparvovec)(Pfizer)*, Hemophilia B

Indication: For adult patients with Hemophilia B due to a nonfunctional coagulation factor IX gene. Methods: Replacement of nonfunctional Factor IX genes using spark therapeutics' AAV vector platform.

In clinical practice today, gene therapy for the treatment of hematologic cancers is still relatively uncommon. The combination of gene and immunotherapy approaches is likely to be the most effective way to induce durable remissions for patients with hematologic malignancies.

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