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Abstract. In strict sense gene therapy could be defined as disease treatment by entering genetic material in affected tissue. This definition also includes numerous genetic manipulations such as insertion of cloned genes or parts of genes, genes from other genomes, artificial genes, antisense oligonucleotides etc. At the moment gene therapy is limited exclusively to somatic cells. Basic common strategies in gene therapy are: gene augmentation or supplement, gene suppression and targeted killing of specific cells. New directions in the development of gene therapy strategies are: replacement of affected gene with normal copy, treatment with antisense oligonucleotides and direct reparation of gene mutation. Gene therapy could be ex vivo or in vivo, using viral or non viral vectors for delivery of the material. At this moment therapeutic transfer of genetic material still faces several major technical limitations. However, there is a hope that in the near future this way of disease treatment will become a part of routine medical practice. Muscular dystrophies as inherited monogenic disorders are on the top of diseases -candidates for such type of treatment. In Duchenne muscular dystrophy (DMD), classic approaches with gene transfer by viral vectors or direct administration of naked DNA have been
tested years ago, but only in animal models or in culture of human cells. Today, DMD is on the focus because of promising new strategies, such as exon skipping and nonsense codon suppression. Success of the early trials has been encouraging, but real clinical benefit and long term effects should be critically evaluated.

**General considerations of gene therapy**

In a wider sense, gene therapy would entail any exogenous influence on the activities of certain genes. Such examples are thyroid hormones used in the treatment of hypothyroidism or steroid hormones in the treatment of asthma, which are already in the application for years. In strict sense gene therapy could be defined as disease treatment by entering genetic material in affected tissue. This definition also includes numerous genetic manipulations such as insertion of cloned genes or parts of genes, genes from other genomes, artificial genes, antisense oligonucleotides etc. Most often genetic modification is directed to affected cells, but in some cases targets can be healthy cells. An example is treatment of immune cells in order to enhance immune response, which would be a form of vaccination. It should be noted that at the moment gene therapy is limited exclusively to somatic cells (1,2).

Some important criteria for the selection of diseases - candidates for gene therapy as follows: there is no other efficient way of treatment; “therapeutic window” from the disease onset to irreversible changes exists; (primarily) one organ is affected; there is good animal model and successful therapy in human cells in vitro; procedure is safety; for inherited monogenic disorders, causing gene is identified and well characterized (1,2).

There are a few basic common strategies in gene therapy. Gene augmentation or supplement is introduction of an additional copy of normal gene; this approach is appropriate in diseases caused by loss-of-function mutations. Gene suppression is introduction of genetic material that is able to block harmful one, and it is recommended for gain-of-function mutations. Targeted killing of specific cells, with the help of monoclonal antibodies, is another approach. New directions in the development of gene therapy strategies are: replacement of affected gene with normal copy, treatment with antisense oligonucleotides and, as a final goal, direct reparation of gene mutation (1).

There are several ways of gene therapy implementation. Ex vivo therapy means that patient’s cells are isolated, genetically modified, and then returned to the patient. In vivo therapy means that therapeutic gene is introduced directly in the organism (intravenously, by inhalation etc.). In addition, in situ access comprises application of therapeutic genetic material
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(with or without vector) in localized and accessible part of body (skin, muscle) (1,2).

Procedures for genetic material delivery to treated tissue may also be different. Possible efficient vectors for gene transfer into the target cells are viruses. In viral vectors sequences that may be harmful for the host are removed, and therapeutic sequence is inserted in the vacant position. Certain types of viral vectors differ in relation to the size of genes that could brings, affinity for a specific tissue, the ability for being integrated in host genome etc. Most frequently used are retro viruses, adeno and adeno-associated viruses, herpes viruses. There are non-viral vectors also. Such example is liposome, small lipid sphere with captured therapeutic DNA, which easily fuses with cell membrane and releases content into the cell. Great advantage of liposome is that does not raise any immunological reactions, but usually level of therapeutic gene expression is not high enough. The simplest approach to gene transfer is the naked DNA introduction locally in specific tissue or by systemic ways, but success of this procedure is very variable. It has proven that for example in muscles, simple injected DNA without any vector can infiltrate the cells and keep for months. “Gene gun” allows tissue bombing by particles (gold, titanium) loaded with therapeutic DNA (biolistic method) (2,4).

At this moment, therapeutic transfer of genetic material faces several major technical limitations, such as ineffective delivery and low level of long term expression. Besides these issues gene therapy brings with it problems related to side adverse effects, as a consequence of manipulation with genetic material. For example, viral vectors could cause severe even deadly infection in a patient. Also, incorporation of foreign DNA into the patient’s genome may be to launch malignant transformation. Both complications have been recorded in practice in several cases (1,2).

In summary, for a relatively short time gene therapy has come a long way from science fiction to (near) reality. Beside the problems mentioned above, there is a hope that selective manipulations with genes, the replacement of defective gene, inhibition of harmful and unwanted genes as well as activation of genes that will lead to reparations or damaged tissue regeneration, in the near future become a part of routine medical practice (2,3).

An example of Duchenne muscular dystrophy

Molecular basis of Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a severe progressive muscle degenerative disease, occurring in about 1 : 3000-3500 live male births. DMD
is caused by the absence of protein dystrophin due to mutation of corresponding gene located on the X chromosome. Milder allelic form of disorder is Becker muscular dystrophy (BMD), where dystrophin is altered but partly functional, and clinical course is generally benign. Because of X-linked recessive pattern of inheritance, both diseases primarily affect males (5).

The dystrophin gene spans more than 2Mb on Xp21 region and consists of at least 79 exons and several tissue specific promoters. Major muscle promoter is for 13kb transcript, present in skeletal and cardiac muscle tissue. Protein dystrophin is a cytoskeletal protein that belongs to the spectrin superfamily. In the muscle fiber, dystrophin is localized at the inner surface of sarcolemmal membrane forming part of a complex protein axis that connects cytoskeleton, sarcolemmal membrane, and extracellular matrix. Muscular dystrophin consists of four morphological and functional domains: N-terminal, rod like, cysteine-rich, and C-terminal domain. Dystrophin alterations are mostly due to intragenic deletions, accounting for 60–65% of DMD/BMD cases (5,6).

According to the reading-frame rule, DMD is caused by frame-shifting mutations and consecutive synthesis of truncated, non functional dystrophin. On the contrary, BMD is associated with in-frame gene mutations and maintained synthesis of dystrophin. DMD begins in the early childhood and has lethal outcome after 2-3 decades of progression. Generally, BMD has late onset and relatively slow progression rate, but considerable clinical diversity exists. Phenotypic effects of in-frame deletions in BMD mostly depend on its relative localization along the gene. Deletions localized at distal hot-spots (exons 44–60) are mainly associated with classical BMD, while proximal deletions (exons 3–13) tend to have various manifestations. Moreover, some patients with large in-frame mutations remained asymptomatic until late 60s years of age (5,6,7).

Due to broad clinical spectrum of BMD, determination of dystrophin gene deletions (localization and extension) and correlation with patient’s phenotype is very important (8). Such studies provide data about specific roles of different parts of the dystrophin gene and the protein itself, with possible therapeutic implications (6,7).

**Classic gene therapy of Duchenne muscular dystrophy**

Knowledge of the molecular basis of DMD, clinical observations of mild BMD phenotypes, as well as experiences with animal model of dystrophic phenotype – mdx mouse have been used for identifying potential DMD therapeutic strategies.
Initially, it seemed that gene therapy by viral vectors has good potential to restore dystrophin in DMD muscles. First major issue for the implementation of this strategy is extremely large size of the dystrophin gene. Several types of dystrophin mini-genes have been constructed in order to solve this problem. In vivo models of effective mini-genes are those with large in-frame deletions associated with mild BMD phenotype. Several studies in mdx mice or in human muscles in vitro showed successful transfer of dystrophin mini-genes, but with only short-term expression. Another approach focuses defect of sarcolemma that occurs due to lack of dystrophin and its associated proteins. Phenotypic improvement has been accomplished by upregulation of compensatory proteins (i.e. utrophin, integrin-a7, or sarcospan); chemical repair of the damaged membrane (i.e. poloxamer); and increased glycosylation of α-dystroglycan to improve extracellular matrix attachment. Although these therapeutic approaches seemed as great promises they have not proceeded to the phase of clinical investigation (9,10,11).

Two recently developed emerging therapies target directly defect of the dystrophin gene and they are in phase I, II or III clinical trials. These agents generate a functional or partially functional dystrophin protein by ‘exon skipping’ and ‘nonsense codon suppression’, respectively.

**Exon skipping**

Majority of DMD cases are due to out-of-frame intragenic deletion in the dystrophin gene. Central idea of exon skipping is restoration of dystrophin’s reading frame by the removal of a targeted exon(s) from the mature RNA transcript. This will restore (a partially functional) dystrophin protein with expected clinical benefit. These expectations are based on number of data that large, in-frame deletions within the dystrophin rod domain could exhibit a milder clinical phenotype consistent to Becker muscular dystrophy (BMD) (12,13). These observations are consistent to classic Monaco’s model of DMD vs. BMD molecular genetic base (5). In addition, in vivo ‘exon skipping’ in the mdx mouse and in humans with DMD has been observed. All these data indicated restoration of the dystrophin proper reading frame by modified RNA splicing as a reasonable therapeutic strategy.

Therapeutic exon skipping till now has been tested in animal models of dystrophin deficiency and in human DMD trials. Antisense oligonucleotides (AONs) are designed to direct the lack of inclusion of targeted frame-shifting exons into the translated mRNA. The feasibility of exon skipping was demonstrated more than 10 years ago with successful administration of oligonucleotides and induction of RNA modification in mdx mice in vivo. In the following few years successful systemic administration in the mdx
mouse was done, but therapeutic levels of the efficiency was not reached yet. Till now various chemistries and delivery methods have been tested in the mdx mouse; many researchers have continued investigations, having in mind possible toxicity and immunogenicity of applied molecules. For example, phosphorodiamidate morpholinos coupled to Arg-rich, cell-penetrating peptides effectively restored dystrophin in 96% of mdx skeletal muscle fibers, but with less efficiency in cardiac muscle (58%). Other studies using octaguanidine coupled morpholinos showed improved delivery with this modification. Administration of 2-O-methyl oligonucleotides to mice for 8 months has been associated with continued phenotypic improvement, suggesting that this approach may be tolerated for extended periods of time. This is very important feature for a chronic disease such as DMD (2,14).

In order to improve AONs delivery, researchers have coupled the oligonucleotides to various carriers. In mdx mice agents such as nanopolymers of polyethylene glycol and polyethyleneimine and polylactide-co-glycolic acid nanoparticles and cationic core shell nanoparticles were used, but all of these studies will require additional exploration of their potential toxicities (14).

As alternate approach, AON cloning in adeno-associated virus (in tandem with a modified U7 small nuclear RNA sequence) has been proposed. This classic-gene-therapy-like approach has its attendant problems. However, the possibility of a more permanent repair without the need for continued therapy is very attractive. Improvements in this process have recently been published (2,10,14).

It is not clear how small antisense sequences interfere with RNA splicing, which is complex process involving numerous proteins and splice enhancer sequences. In addition, each DMD deletion has unique length and breakpoints and optimal specific sequence to target is not always clear. In any case prior to clinical trials safeties of used therapeutic intervention should be documented at the animal model and human cells in culture (2,14).

Exon 51 is the most common single exon skipping target in DMD patients. This exon affects reading frame in about 13% of DMD cases, including frequent deletions such as exon 47–50, 48–50, 49–50, 50, and 52. For this reason the first human clinical trials are focused on exon 51, and AONs that efficiently induce frame restoration have been identified. Trials have been conducted in Europe using two different chemistries. In the Netherlands, researchers administered agent called PRO051 into the tibialis anterior muscle of four DMD patients. In each patient muscle biopsy showed detectable levels of dystrophin protein without adverse effects. This successful in vivo exon skipping was a key landmark for proof of-principle studies in humans. Phase I/II studies using systemic administration of
PRO051 by subcutaneous delivery are underway and will test the safety and efficacy of a 5-week treatment regime and 13 weeks of follow-up. In parallel, local introduction of AVI-4658, which also targets the same region of exon 51 through alternate backbone chemistry called morpholino, into the extensor digitorum brevis muscle was tested over a year ago and results indicate that some dystrophin expression was restored in the injected muscle. Similarly, a 12-week, phase I/IIa systemic delivery clinical trial of AVI-4658 has been initiated at Imperial College London (2,14).

Following exon 51, the next six most common single exon skip targets are exons 45, 53, 44, 46, 52, and 50. Recently, two studies have demonstrated that exon skipping can also be used with complicated dystrophin mutations that lead to ‘pseudoexons’. It seems that different types of deletions as well as some point mutations may benefit from this type of therapy.

Some investigators test the feasibility of developing a cocktail of AONs, which could be used as a single drug to treat as many as 63% of all patients with DMD. Results of animal studies encourage this approach. Cocktails of 2-O-methyl oligonucleotides against all exons between 45 and 55 were tested in human cells in vitro. Unfortunately, the researchers were not able to identify a cocktail that was effective for inducing such a large deletion; thus, additional studies will be necessary before therapeutic exon skipping between exons 45 and 55 becomes a reality (14).

These preliminary studies demonstrate that exon skipping is a viable strategy to induce the production of dystrophin in DMD boys. However, it is unclear whether the levels of skipped dystrophin currently being achieved will be sufficient to functionally reverse the disorder, particularly in DMD boys. Best estimates indicate that 30–60% of wild-type levels of skipped dystrophin will be required to functionally compensate for loss of dystrophin. Early trial data, though promising, indicate that even high-dose local intramuscular delivery of AON yields only 3–35% of normal dystrophin. It is anticipated that systemic delivery of AON may be even more inefficient. Therefore, it will be imperative to increase the efficacy of exon skipping to replace dystrophin to functionally relevant levels, which is being pursued by altering the oligonucleotide backbone, altering the targeted sequence, modifying attachments to the oligonucleotide sequence, increasing delivery to muscle, and identifying small molecule facilitators of exon skipping (2,14).

These observations are consistent with most recent reports of AONs clinical trials about experimental drug drisapersen. This agent, in development to treat boys with DMD who have mutations near exon 51 of the dystrophin gene, did not show benefit on tests of walking distance or motor function in a 48-week, phase III trial that included 186 participants.
Full evaluation of effects of drisapersen treatment across all conducted studies should be done (2,14).

**Nonsense codon suppression**

At least 25% of all DMD causing mutations in the dystrophin gene are substitutions classified as nonsense mutations. These mutations create premature stop codon and result in a truncated, nonfunctional protein. Several years ago it was demonstrated that aminoglycoside antibiotics have the capacity to reduce ribosomal fidelity for recognizing these premature termination codons (PTCs) in the dystrophin RNA transcript. Through this mechanism, aminoglycosides induce ribosomal read-through of premature termination signals. This mechanism results in the generation of a full-length dystrophin protein with only one amino acid substitution, which corrects the primary genetic defect. Exposure of mdx cells or mice to the aminoglycoside gentamycin induced read-through of the PTC in exon 23 of the dystrophin transcript and production of dystrophin protein. This study was the first successful demonstration of pharmacological correction of a primary genetic defect in vivo and provided a proof-of-principle that such a therapy held promise. Unfortunately, these antibiotics were known to be too toxic for long-term therapy and were relatively inefficient. Subsequently, a screen of 800 000 compounds was conducted against a luciferase reporter that harbored a UGA premature stop codon. Through the screen and additional chemical modifications, a lead compound called PTC124 (ataluren) was identified (2,14).

PTC124 has proven to be efficacious in mdx mice and to some extent in clinical trials for both DMD and cystic fibrosis. Phase I and IIa clinical trials demonstrated good safety and tolerability in DMD boys and phase IIb clinical trials are fully enrolled with over 165 individuals. There is apparently little toxicity from the oral drug, though efficacy in protecting DMD has yet to be established. According to the first published results, levels of dystrophin induced by PTC appear to be 35% and 40–60% of normal in mouse and human, respectively, within the range predicted to be necessary for functional improvement. Some caution that longer term exposure to nonsense codon suppression could permit reactivation of effectively silenced retrotansposons. Although PTC124 targets a minority of DMD mutations, if successful it has the potential to be a substantial treatment for a subset of DMD patients. PTC124 is not specific to the gene but rather to the type of mutation, and it has the potential to be efficacious in many other recessive disorders that commonly include nonsense mutations (2,14).
Conclusion

Gene therapy is one of the challenges and hopes of the biomedicine in the beginning of the XXI century. Muscular dystrophies as inherited monogenic disorders are on the top of diseases - candidates for such type of treatment. In Duchenne muscular dystrophy (DMD), classic approaches with gene transfer by viral vectors or direct administration of naked DNA have been tested years ago, but only in animal models or in culture of human cells. At the moment, DMD is on the focus because of promising new strategies, such as exon skipping and nonsense codon suppression. Success of the early trials with several experimental drugs has been encouraging, but real clinical benefit and long term effects should be critically evaluated.

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References


