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REVIEW ARTICLE

An introduction to gene therapy and its potential prenatal use

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This review article is an introduction to the general field of gene therapy with examples from the specialized field of prenatal gene therapy.

Key words: gene therapy; molecular biology; prenatal; vectors

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Definition of gene therappy

Gene therapy has been defined as 'nucleic-acid-based treatment, or transfer of DNA/RNA to somatic target cells in the intention to treat serious illness' (1). In somatic gene therapy, new genes are introduced into body (somatic) cells. The therapy will only affect the treated person, and not future generations. In germ cell therapy, the human germline is modified, and the genetic alterations would be transferred to offspring. The latter is not considered ethical, and is not permitted in any country in the world.

Simple basics of molecular biology

A basic knowledge of molecular biology is necessary to understand the essential of gene therapy (reviewed in: 2). The genetic information of the DNA molecule is based on the sequence of the 4 different bases along the DNA molecule: adenine (A), thymine (T), guanine (G), and cytosine (C). Each base on a DNA strand binds by hydrogen bonds to the base on the opposite DNA strand, making up a basepair. A will only bind to T, and G only to C. In this way two complementary DNA strands are formed. The information in the DNA is transformed into production of proteins. A gene is a part of the DNA molecule containing all the necessary bases coding for the amino acids that build a particular protein. There are about 3 000 million basepairs in the human DNA-molecule and this genetic information is divided into the 46 chromosomes that are found in each of the body cells. The human DNA molecule in each body cell contains about 100,000 genes. A single mutation, a wrong letter in the DNA molecule, can result in disease, such as in cystic fibrosis.

During transcription, the genetic information from DNA is passed on to the single-stranded mRNA messenger RNA (*r*ibo*n*ucleic *a*cid), which can pass into the cytosol of the cell. At the beginning of each gene there is a base sequence called the gene promoter, which starts the production of the mRNA molecule (transcription of the gene) by binding the enzyme RNA polymerase. The induction or start of gene transcription is a very compli-

Abbreviations:
A: adenine; ADA: adenosine deaminase; AIDS: acquired immune deficiency syndrome; C: cytosine; cftr: cystic fibrosis transmembrane receptor; CMV: cytomegalovirus; DNA: deoxyribosenucleic acid; G: guanine; HIV: human immunodeficiency virus; MAC: mammalian artificial chromosome; mRNA: messenger RNA; RNA: ribonucleic acid; SCID: severe combined immunodeficiency disorder; T: thymine; VEGF: vascular endothelial growth factor.

cated event where many elements cooperate, such as transcription factors, nuclear receptors, regulatory DNA elements, coactivators, and corepressors. In the cytoplasm, this genetic information in the mRNA molecule is translated into the formation of proteins (translation) on the ribosomes. Three bases on the mRNA molecule attract a transporter molecule with one of the 20 possible amino acids the cell uses to build up the proteins. By determining protein production, the genes regulate essential cell functions. Proteins are involved in transporting energy sources in the form of lipoproteins; they are involved in the energy metabolism (hormones are proteins), and in cell receptors etc.

Current status of gene therapy

The first human gene transfer was performed 10 years ago. Since then, more than 400 clinical protocols have been approved. Approximately 3–4000 patients have received gene therapy, mostly cancer patients (69%), HIV (Human Immunodeficiency Virus) positive AIDS (acquired immune deficiency syndrome) patients (11.5%), and patients with genetic diseases such as cystic fibrosis and certain immunodeficiencies (16.5%) (3). Large patient groups with cardiovascular and neurological diseases are also emerging as targets for gene therapy. As of January 2000, three patients have been treated with gene therapy in Norway, and these patients were included in international multicenter studies (1).

Until now, only a few patients with rare conditions have benefited from gene therapy. Gene therapy is not an established treatment today, except for cytomegalo-virus eye infection in AIDS patients (1). However, there is intense research going on to improve gene therapy as a therapeutic method. There is still great optimism as to the eventual clinical success of gene therapy, although it is hard to predict exactly when it will happen (4). Until now, no human prenatal gene therapy trials have been performed, but two pre-protocols have been suggested, and animal studies are progressing.

Essential to gene therapy is the mapping of the human genome, the DNA. Genetic diseases and cancer are caused by mutations, and to be able to correct the mutations, the normal base sequences need to be known. Despite the recently finished sequencing of our genes by the Human Genome Project and its commercial competitor Celera (5), there are still many unanswered questions. Many diseases have heterogeneous causes, and are also influenced by environment (including food intake, exercise, drugs etc).

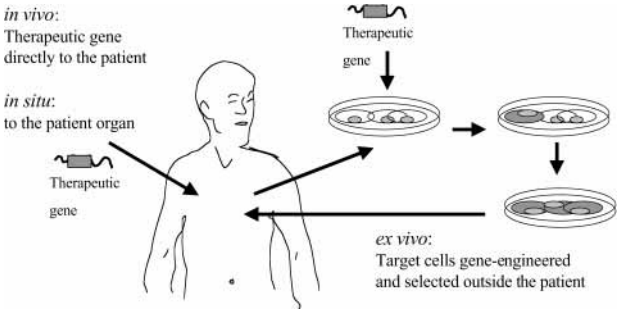

Fig. 1. In vivo gene therapy is the transfer of therapeutic genes directly to the patient, for example by injection into the blood stream. *In situ* gene therapy is a specialized form of *in vivo* therapy, where the therapeutic gene is directly injected into a selected tissue of the patient (for example muscle tissue). *Ex vivo* gene therapy involves transfer of therapeutic genes into cells grown in culture. These cells are first removed from the patient. The cells that are successfully genetically engineered are selected and expanded in culture before being returned to the patient.

In vivo/*ex vivo* gene therapy

There are two main approaches to gene therapy: *in vivo* and *ex vivo* gene therapy (6) (Fig. 1). *In vivo* gene therapy is defined by delivery of new genetic material directly to target cells in the body, often injected into the patient's blood stream. The advantage of the *in vivo* approach is that the injection procedure is simple, but the challenge is to reach the correct target cells in the body. This method therefore still needs to be improved before clinical trials in humans. *In situ* gene therapy is a specialized form of *in vivo* gene therapy where the new genetic material is placed directly into the affected tissue. Examples are injection into a tumor mass with a vector carrying the gene for a toxin, or the injection of a vector carrying a dystrophin gene directly into the muscle of a patient with muscular dystrophy (3).

Ex vivo gene therapy is where target cells are removed from the body and then genetically modified outside the body (Fig. 1). The cells are returned to the body after selection and amplification of the modified cells. The *ex vivo* approach is safe because the appropriate cells can be selected in culture, but this method can only be used in diseases where the target cells can be removed from the patients (e.g. blood cells).

How can gene therapy alter the function of a cell?

Proteins are essential in most cell functions, and gene therapy is aiming at altering protein expression (reviewed in: 1, 7). Many of the gene therapy principles still have a long way to go before proving successful in clinical trials.

Gene replacement is a method where a non-active or defective gene is substituted by a 'new' or additional functional copy of the gene. It restores the production of a required protein (for example in cystic fibrosis restoring production of cftr-cystic fibrosis transmembrane receptor). With this method, defects in a gene are replaced by a normal copy at the DNA level. This can be done for instance by homologous recombination where the defect part is exchanged with the correct version that is inserted into the cells. This method needs to be improved since the efficiency of this homologous recombination is extremely low. Recently, hybrid molecules of DNA and RNA, called chimeras, have been shown to trick the cell's DNA repair machinery into replacing the wrong nucleotide in a gene with the right one, representing a potential new route to gene therapy (8).

An additional way of altering cell function by gene therapy is by gene addition, where a new gene is inserted into the cells to enable production of a protein not normally expressed by that cell. An example of this method is the insertion of a 'suicide gene' in cancer cells, coding for a protein that converts a non-active pro-drug into an active drug to kill tumor cells (9).

Yet another general gene therapy method is not to alter the gene itself, but to introduce an alteration or control of expression of a gene. This can be done at the level of transcription, by interfering with the promoter region of the gene, thus inhibiting the formation of mRNA of that gene (7). At the mRNA level, a gene-specific antisense or ribozyme molecule can bind mRNA and thereby prevent specific protein production. An antisense oligonucleotide is a short piece of DNA (15–30 bases often) which is complementary to a small part of the mRNA molecule with which one wishes to interfere. When the antisense binds its complementary mRNA piece, a short part of this mRNA will be double stranded, and the short double-stranded area will stop the translation to protein on the ribosomes. With this method, the gene itself might be turned on, but the protein is not produced. The ribozymes have, in addition, the ability to cut the target RNA molecule by enzymatic action, and the RNA is inactivated.

The protein level can also be affected by gene therapy. Therapeutic genes can code for intracellular antibodies (named intrabodies), or other molecules that can inhibit the function of a specific protein. There are also strategies named decoy strategies, where double-stranded DNA oligonucleotides are transfected into the cells, for example with the intention to remove transcription factors that are necessary to start the transcription of the gene. Also, decoy strategies at the level of translation have been used, removing proteins necessary for translation (10).

Vectors

A carrier molecule, named vector, is used to increase the gene-transfer efficiency in gene therapy, since only a small amount of naked DNA will pass into the cell. The optimal vector would be a vector that does not give an immune response, that could be targeted to specific tissues for long-lasting gene expression, and where a regulation of the gene activity could be obtained (11).

The vectors used in gene therapy can be divided into viral and non-viral systems. About 20% of human gene therapy trials have used non-viral systems (3), but, in general, non-viral vectors have much lower gene transfer efficiency than viral vectors. In non-viral vector systems, there is no integration of introduced DNA, and therefore a transient expression of the therapeutic gene. Non-viral vectors include: direct transfer of therapeutic DNA into target cells (gene gun, direct DNA injection), liposomes (13), receptor mediated endocytosis (13), and mammalian artificial chromosomes (MAC). MAC do not require insertion into the genome and could include sufficient genomic sequences to ensure proper tissue-specific and temporal regulation. So far, limited success has been obtained in human cells (14).

Many different viruses have been tried as vectors in gene therapy. By removing the viruses' own viral genes, space is made for insert of the new therapeutic gene, and the virus is rendered unable to

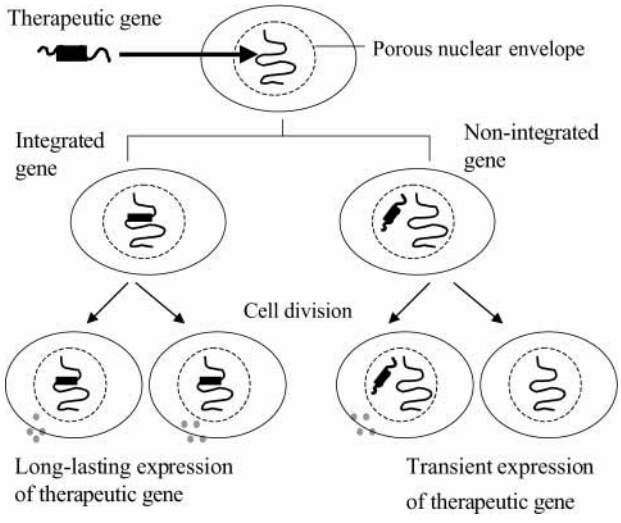

Fig. 2. Integration *versus* non-integration of therapeutic gene, resulting in extended *versus* transient production of therapeutic protein. Therapeutic genes that are integrated into chromosomes can be transmitted to all daughter cells, unlike extrachromosomal genes.

replicate or cause disease in the new host. About 60% of human gene therapy clinical trials have used RNA viruses as vectors (retroviruses and lentiviruses, for instance), whereas 10% have used DNA viruses as vectors (adenoviruses and adeno-associated viruses, for instance) (3). The most common viral vectors used in clinical trials are the retroviruses and the adenoviruses. The major difference between these two systems is that retroviral vectors integrate their therapeutic DNA into the chromosomes of the target cells, whereas adenoviruses do not integrate the therapeutic gene. Fig. 2 shows the difference between integration and non-integration of a therapeutic gene into host cell chromosomes. If a therapeutic gene is integrated, all daughter cells will have the integrated gene when the cells are dividing (S phase of cell cycle). In gene therapy, the advantage of gene integration is stable expression of the inserted gene, which gives a long-lasting production of the therapeutic protein and a possible permanent cure of a disease (7). The concern about gene integration is that one of the integration events may result in disruption of normal genes, and end in cancer. The integration of the new gene occurs almost randomly on the DNA and the new integrated gene could in theory activate a cancer gene (oncogene) or inactivate a tumor suppressor gene. Retroviruses are examples of vectors that integrate their therapeutic DNA into host chromosomes. Genes that are not integrated into the host DNA (episomal genes) replicate extrachromosomally during cell division, and may not be passed on to all daughter cells (7). With each cell division, the percentage of cells expressing therapeutic protein is reduced. In this case, there is a transient expression of the therapeutic protein, and a repeated treatment with gene transfer is often necessary.

A problem with retroviruses, besides the safety concern of random gene integration and possible unwanted affection of normal genes, is that they are only produced at relatively low titers, so it is not possible to get a large number of vector particles to the desired cell type *in vivo*. The result is low gene transfer efficiency. Also, even if the therapeutic gene is integrated, gene expression is often shut down after a while (some days/week). The regulatory sequences that control the genes are inactivated, probably because the cells recognize foreign viral promoters and inactivate them (by methylation or other mechanism). Lentiviruses are new types of retroviruses (single stranded RNA as genome) being evaluated for use in gene therapy, and the HIV (Human Immunodeficiency Virus) belongs to this group (14, 16). An advantage of the lentivirus-based vectors is that both dividing and non-dividing cells can take them up, and the foreign DNA is integrated into the host genome similarly to the other retroviruses.

Adenovirus vectors used in gene therapy are based on adenoviruses, which normally produce infections of the upper respiratory tract (11–13, 16). The adenovirus vectors have several advantages; they can be produced in high titers in culture and they can infect many different human cell types, including both dividing and non-dividing cells. The efficiency of gene transfer is very high, often approaching 100% *in vitro*. Since they are large viruses, they can accept large therapeutic gene inserts. The disadvantage of adenovirus vectors is that the expression of the inserted gene is short and transient due to non-integration of the gene. There is also the risk of infection of non-target cells since they can infect almost all cell types. Most importantly, they can give unwanted immune responses, causing immunological and inflammatory reactions. The first human gene therapy death reported was in September 1999, when a young man with a metabolic disease died after adenovirus based gene therapy, probably caused by an overwhelming immune reaction to the adenovirus. This death caused extensive debate regarding the monitoring of clinical gene therapy trials (17, 18).

Possible future directions of gene therapy

In general, the first decade of clinical gene therapy has shown that more basic research is required before clinical success can be expected (4). Currently, gene therapy is not an established treatment for any disease, except for the treatment of cytomegalovirus eye infections in AIDS patients in the USA, using antisense DNA-oligonucleotides against human CMV mRNA (1).

Diseases with potential clinical benefit in the future include monogenetic diseases, such as ADA (adenosine deaminase) deficiency, cystic fibrosis, and hemophilia. Also diseases with possible alterations in several genes, such as in cancer, are possible candidates for gene therapy (1). There are, for example, high expectations regarding the possibility of improving cancer therapy with suicide gene therapy, aiming at maximizing the effect of a toxic drug and minimizing its systemic effects, by generating the drug *in situ* within the tumor (9).

Gene therapy might also have great impact in the future on the treatment and prevention of the cardiovascular diseases, and some human trials have already been performed. LDL-receptor deficiency, which is a severe inherited genetic defect that may lead to atherosclerosis, might in the future be treated by liver-directed LDL-receptor gene transfer (11). Future applications include revascul-

arization of ischemic tissue by improving angiogenesis, for example with the production of VEGF (Vascular Endothelial Growth Factor), in peripheral vascular disease and in coronary artery disease (reviewed in: 4, 11). Gene therapy strategies with the goal to prevent restenosis following angioplasty are also developing, aiming at inhibiting smooth muscle cell migration and proliferation (11).

Gene therapy strategies in the treatment of infectious diseases, including AIDS, and neurological diseases are currently under evaluation (1, 12, 19). Intense research is also being performed aiming at developing either therapeutic or preventive DNA-based vaccine against malaria, tuberculosis, hepatitis A, B, and C viruses (16, 19).

Prenatal gene therapy

For the field of clinical obstetrics, the possibility of performing gene therapy prenatally is of special interest. Why should gene therapy on a fetus even be considered? First of all, it is believed to be a more efficient gene transfer and permanent integration of foreign DNA in the developing fetus than in a child or adult (20). Fetal stem cells are good targets for gene transfer, they are precursors of the more differentiated cells and organs in the more mature organism. Secondly, the fetal immune system is not completely developed until several months after birth, and this may permit induction of immune tolerance against vector and the therapeutic gene (21), thus avoiding adverse immune reactions. It is believed to be a 'therapeutic window' up to about 14 weeks of pregnancy, and possibly even longer in some cases of abnormal T-cell production. Before the thymus processes mature lymphocytes, the fetus is largely tolerant to foreign antigens. This window is the period before the release of mature T-lymphocytes into the circulation, while the bone marrow is beginning to develop sites for hematopoiesis. One major obstacle in gene therapy after birth is the immune response of the host to the 'foreign' protein encoded by the delivered gene. Therefore, introducing the therapeutic gene prenatally hopefully enables the immature immune system to accept the new protein as 'self'. Thirdly, prenatal gene therapy could be delivered before disease symptoms are irreversible. There are situations where postnatal gene therapy may not be delivered in time to avoid irreversible damage. Examples are some neurologic genetic diseases such as Tay-Sachs, Lesch-Nyhan etc. Fetuses with immunological and metabolic diseases would also benefit from treatment before they have any symptoms of the disease. Fourthly, obstetrical ultrasound technology is well developed with high-resolution ultrasound and intervention techniques, enabling gene transfer to the mother and the fetus. In families at risk, evaluation of trophoblastic tissue obtained by chorionic villus sampling in the first trimester of pregnancy may allow early identification of a genetic disorder in the fetus (20).

It has been argued that to treat a disease prenatally, it should be a life-threatening disease, where there must be a clear advantage over postnatal gene therapy, and where no other reasonable treatment is available. It is also best if the expression of the therapeutic gene does not need fine regulation to obtain effect of the treatment, because a tight gene regulation would be complicated to achieve. Also, target cells or organs must be accessible in the fetus, and this is a limitation today, for instance for the treatment of neurological genetic diseases prenatally (22).

Over 4000 single gene disorders are now known, where defects in one gene function only is responsible for the disease. In general, recessively inherited disorders are the easiest inherited disorders to treat by gene therapy. High level expression of an introduced normal allele should be sufficient to overcome the genetic deficiency. In recessively inherited disorders the mutations are almost always simple loss-of-function mutations, and the disease phenotype is due to non-functioning gene products from both alleles. Heterozygotes have 50% production of normal gene product and are normally without symptoms. Since the level of gene product inversively correlates with the severity of the disease, even modest expression of the introduced gene may make a substantial difference. In contrast, in dominantly inherited disorders, the heterozygotes have 50% of the normal gene product and yet may be severely affected due to expression of the mutant gene, which interferes with the correct one or gains a new function.

Many different gene transfer strategies have been used in animal studies of prenatal gene therapy in the last ten years. The animals used have mostly been mice, rats and sheep, and many of these animal strategies could be adapted to humans, and both *in vivo* and *ex vivo* strategies have been used. The routes where the therapeutic gene material has been delivered have been through the amniotic cavity surrounding the fetus, the placenta, or into the fetus itself; for example into the peritoneum, the brain, or vessel walls. An example of *in situ* gene therapy in lambs, that is potentially interesting also for humans, is a study published in *Nature Medicine* in 1999 by Mason et al. (10). The background for this study is that newborns with congenital heart defects involving severe right- or left-sided obstruction of the heart need to maintain an opening of the ductus art-

eriosus after birth, before surgery. This is usually done by giving the newborn baby prostaglandins, but these prostaglandins may have serious side effects, such as hypotension, cardiac arrhythmias, and hypoventilation. Mason et al. inhibited fibronectin protein formation and thereby the closing of the ductus at birth by injecting liposomes with the therapeutic genetic material coding for a decoy RNA directly into the walls of the ductus arteriosus, resulting in inhibition of translation of fibronectin mRNA into protein. At birth, the ductus arteriosus was still open in the lambs that had received the gene therapy prenatally (10). *Ex vivo* gene transfer to the placenta is an example where there is avoiding of direct gene transfer to the mother or fetus. In a study by Senut et al., placenta cells were removed, infected with the therapeutic gene, and reimplanted into the placenta of other pregnant rats (23). The therapeutic protein was produced by the gene-engineered placenta cells and transferred to the fetus by the umbilical cord. With this approach, the fetus would receive the gene product substances only until birth when the placental-fetal connection is lost. This example of prenatal placental gene therapy may provide a new therapeutic approach to the treatment of diseases with pathologic onset during early embryonic life (23).

Two pre-protocols for human prenatal gene therapy were presented in July 1998 by French Anderson, director of the Gene Therapy Laboratories and Professor of Biochemistry and Pediatrics at the University of Southern California (USA), intended at promoting a discussion regarding the start of future prenatal human gene therapy (20). His suggestions for the first diseases to treat prenatally were the hematologic disease α-thalassemia caused by deletions of the α-globin gene and a severe combined immunodeficiency disorder (SCID) caused by deficiency of the adenosine deaminase (ADA) enzyme due to mutations in the ADA gene. These two disorders were chosen because the regulatory sequences of the globin gene and the ADA gene are well characterized. One of the obstacles to obtaining permanent effect of gene therapy is that the new therapeutic gene is shut down after a while (days/weeks). This is presumably because of an inactivation of the foreign vector virus promoter, either by immune response or from transcriptional silencing (by methylation of regulatory sequences for example). Therefore, it is preferable if the retroviruses could carry the human variants of regulatory sequences of these genes to give a long lasting gene expression. The target cells are the fetal hematopoietic stem cells, which are rapidly dividing during fetal development and therefore can be infected with retrovirus vector strategies. The therapeutic genes are the α-globin gene in the case of α-thalassemia and the normal ADA gene in fetuses with ADA deficiency. In theory, prenatal gene therapy could transform enough stem cells to allow the fetus to survive to delivery and have no postnatal symptoms of the disease. It has been argued that there are better candidates for the start of human prenatal gene therapy than ADA deficiency, because there are alternative treatments after birth for this disease. There are other immunodeficiencies that might be better choices (24). The plan of French Anderson and his team, when putting forward the pre-protocols on α-thalassemia and ADA deficiency, was to work on transfer techniques in small, then in larger animals, and report to the RAC (Recombinant DNA Advisory Committee) every 12 months. The time-schedule in 1998 for the start of the first human gene therapy was 3 years (24). The presentation of these pre-protocols has generated an animated debate on prenatal gene therapy (21). A British expert committee concluded in 1998 that human prenatal gene therapy was not likely to be acceptable in the foreseeable future, in view of the safety and ethical difficulties (25), similar to the conclusion made recently by an official Norwegian expert committee on gene therapy (1).

The main argument against starting human prenatal gene therapy is that human gene therapy has not worked very well on adults or children yet, and that progress here should be awaited before extending gene therapy prenatally. In addition, potential adverse effects of gene therapy can affect two patients, both the fetus and the mother. Another general contra argument not specific for prenatal gene therapy, is the risk of unwanted germline transformation. Also, there is the feared risk of introducing cancer with retroviral vector strategies. It has also been argued there are alternative ways to become parents than by having one's own children. Instead of genetic manipulation prenatally, it has been proposed that couples at risk of having a child with genetic disease can adopt, or be foster parents, or have preimplantation diagnosis. If the preimplantation diagnosis of genetic diseases is more successful and available (it is not currently permitted in Norway) in the future, it would be a better alternative than prenatal gene therapy, since only healthy embryos would be implanted. Human preimplantation gene therapy is not permitted due to affection of both the somatic cells and the germ line, but it could theoretically correct the genetic disorder in all the cells of the body and in future generations. Also, it has been argued that selective abortion is a better option than prenatal gene therapy in a fetus with genetic disease (26).

There are important ethical dilemmas that are special to human prenatal gene therapy. There is the risk of having a child dying after birth instead of prenatally (24). Also, there are the legal and moral rights of the mother *versus* the fetus to be considered. Which limits will there be for altering non-lethal diseases or phenotypes if we know how to manipulate them in the future? What if gene therapy is available for a serious disease and the mother refuses to have any prenatal gene therapy performed, even if it was at low risk in the future?

Conclusions

In conclusion, prenatal gene therapy is still not ready for human clinical trials; it is still at the level of animal research. There are many ethical questions to be solved, the safety of fetus, mother, and future generations needs to be secured. More advances in postnatal gene therapy need to be obtained before prenatal trials start; both regarding vector delivery efficiency, duration of expression of the therapeutic gene, and organ specificity. The safety issues regarding unwanted germ line affection, immunological responses, and possible development of cancer also need to be further evaluated.

In the future, there will most likely be a merging of clinical medicine and molecular science, incorporating genetics in all clinical specialties (27). This will require a close cooperation between molecular biology and clinical medicine. Well-advanced ultrasound technique is a clinical contribution enabling, at least theoretically, non-surgical prenatal gene transfer. Although the field of gene therapy in general, and prenatal gene therapy specifically, needs more basal scientific research and maturation before clinical success is obtained, there is a need for clinicians to understand the basics of the molecular therapeutic possibilities that are emerging in the new millenium.

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