

Nucleic Acids as Therapeutics

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Contents

1	Introduction	20
2	DNA-Based Therapeutics	22
2.1	Plasmids	22
2.2	Oligonucleotides for Antisense and Antigene Applications	23
2.3	Aptamers	23
2.4	DNAzymes	23
3	RNA-Based Therapeutics	24
3.1	RNA Aptamers	24
3.2	RNA Decoys	24
3.3	Antisense RNA	25
3.4	Ribozymes	25
3.5	Small Interfering RNAs	26
3.6	MicroRNA	27
4	Gene Transfer Technologies	27
4.1	Mechanical and Electrical Techniques	27
4.2	Vector-Assisted Delivery Systems	28
5	Current Status of Gene Therapy Research	32
6	Recent Developments in Gene Therapy	33
	References	36

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Abstract Gene therapy is a technique for correcting defective genes responsible for disease development. Nucleic acid-based molecules (deoxyribonucleic acid, complementary deoxyribonucleic acid, complete genes, ribonucleic acid, and oligonucleotides) are utilized as research tools within the broad borders of gene therapy and the emerging field of molecular medicine. Although most of the nucleic acid-based drugs are in early stages of clinical trials, these classes of compounds have emerged in recent years to yield extremely promising candidates for drug therapy to a wide range of diseases, including cancer, infectious diseases, diabetes, cardiovascular, inflammatory, and neurodegenerative diseases, cystic fibrosis, hemophilia, and other genetic disorders. Gene therapy may be classified into two types: somatic and germ line gene therapy. There are many ethical, social, and commercial issues raised by the prospects of treating patients using gene therapy. This chapter summarizes deoxyribonucleic acid-based therapeutics, ribonucleic acid-based therapeutics, and gene transfer technologies. Deoxyribonucleic acid-based therapeutics includes plasmids, oligonucleotides for antisense and antigene applications, deoxyribonucleic acid aptamers, and deoxyribonucleic acidzymes, while ribonucleic acid-based therapeutics includes ribonucleic acid aptamers, ribonucleic acid decoys, antisense ribonucleic acid, ribozymes, small interfering ribonucleic acid, and micro ribonucleic acid. This chapter also includes current status of gene therapy and recent developments in gene therapy research.

Keywords Gene therapy • Nucleic acid therapeutics • DNA-based therapeutics • RNA-based therapeutics • Gene transfer technology • Viral vectors • Nonviral vectors • Liposomes

1 Introduction

Modern drug research aims to discover biologically active molecule(s) that are absolutely specific to the molecular targets responsible for the disease progression. Moreover, there is strong belief that medicine will soon benefit from the development of new therapeutic technologies to directly target human genes. Insertion of new genetic material into the cells of an individual with the intention of producing a therapeutic benefit for the patient is human gene therapy (Anderson 1992; Baltimore 1988; Mizutani et al. 1995), while gene therapy is a technique for correcting defective genes responsible for disease development. Numerous gene therapy strategies are under development, some of which use nucleic acid-based molecules to inhibit gene expression at either the transcriptional or posttranscriptional level (Gewirtz et al. 1998), and this strategy has potential applications, such as in cardiovascular (Mann et al. 1999a, b; Ehsan et al. 2001; Stull and Szoka 1995; Patil and Burgess 2003) and inflammatory disorders (Dean et al. 1994; Yacyshyn et al. 1998), cancer (Mardan et al. 2002), neurological disorders (Shi et al. 2001), and infectious diseases (Macpherson et al. 1999; Welch et al. 1998; Zu Putlitz et al. 1999; Campagno et al. 1999), as well as in organ transplantation (Katz et al. 1997).

A number of human diseases are known to be genetic in origin (e.g., Huntington's chorea and cystic fibrosis), and virtually all diseases, except for some trauma, have a hereditary component (SoRelle 2000). Thus, gene therapy represents an opportunity for the treatment of genetic disorders in humans by modifying their cells genetically (Report 1995).

Despite the obvious advantages that might be gained from human gene therapy (i.e., replacing a defective gene with a normal one), there are many ethical, social, and commercial issues surrounding the technology. The outcome of an error in technology might not be observed for many years. Moreover, it is feared that unpredictable and perhaps irreversible side effects occur in treated individuals. The social implications of such technology include the possibility that patients might suffer from depression as a result of being "genetically altered" or might not be accepted by society in the way that they were before treatment. The commercial implications of such technology are that the insurance companies and other such institutions also would want to access the available information prior to them granting life insurance policies, etc. Hence, it is obvious that a person shown to have a predisposition to a genetic disease could be severely penalized because of a mutation in their DNA.

The possibility of using nucleic acids as drugs for the treatment of genetic diseases is still very much in its infancy. One reason for this is that, unlike monogenetic disorders such as severe combined immune deficiency (SCID), which is caused by a mutation in the adenosine deaminase (ADA) gene, very few diseases are caused by a single gene mutation; most are caused by the mutation of multiple genetic components. For example, cancer usually involves multiple genetic lesions within the same cell and it is unlikely that the nature of every one of these oncogenic mutations is yet known.

Elucidation of the human genome has also provided a major impetus in identifying human genes implicated in diseases, which may eventually lead to the development of nucleic acid-based drugs for gene replacement or potential targets for gene ablation (Baker 2001). Moreover, the Human Genome project will help determine genetic markers responsible for patient response to drug therapy, drug interactions, and potential side effects (Van Ommen et al. 1999). Currently, all gene therapy trials approved for human use target somatic cells that will live only as long as the patient, and this is known as the somatic gene therapy. Its purpose is to alleviate disease in the treated individual alone. In contrast, it is also possible to target directly the gametes (sperm and ova) to modify the genetic profile of the subsequent generation of unborn "patients." This gene transfer at an early stage of embryonic development is known as the germ line gene therapy. More than 300 clinical trials involving gene transfer in patients have been approved, and the first nucleic acid drug, an antisense oligonucleotide, fomivirsen (marketed as Vitravene), has been approved by the US Food and Drug Administration (FDA) for the treatment of cytomegalovirus retinitis in immunocompromised patients (Rosenberg et al. 2000).

In 1999, the biggest setback for gene therapy occurred when Jesse Gelsinger, an 18-year-old high-school graduate from Arizona, died as a result of a gene therapy

experiment. Gelsinger developed fever and blood clots throughout his body within hours of treatment to correct partial ornithine transcarbamylase (OTC) deficiency, a rare metabolic disease that can cause a dangerous buildup of ammonia in the body, and died 4 days later (Lehrman 1999). The FDA has not yet approved any human gene therapy product for sale.

This chapter summarizes DNA-based therapeutics, RNA-based therapeutics, and gene transfer technologies for the diseases that are known to be genetic in origin. DNA-based therapeutics includes plasmids, oligonucleotides for anti-sense and antigene applications, DNA aptamers, and DNAzymes, while RNA-based therapeutics includes antisense RNA, ribozymes, RNA decoys, RNA aptamers, small interfering RNA, and microRNA. This chapter also includes the current status of gene therapy and recent developments in gene therapy research.

2 DNA-Based Therapeutics

2.1 *Plasmids*

Plasmids are high molecular weight, double-stranded DNA constructs containing transgenes, which encode specific proteins. On the molecular level, plasmid DNA molecules can be considered prodrugs that upon cellular internalization employ the DNA transcription and translation apparatus in the cell to biosynthesize the therapeutic entity, the protein (Uherek and Wels 2000). The mechanism of action of plasmid DNA requires that the plasmid molecules gain access into the nucleus after entering the cytoplasm. Nuclear access or lack thereof eventually controls the efficiency of gene expression. In addition to disease treatment, plasmids can be used as DNA vaccines for genetic immunization (Johnston et al. 2002). In the early stages of development, plasmid-based gene therapy was attempted to correct inheritable disorders resulting from a single gene defect. The first federally approved human gene therapy protocol was initiated in 1990 for the treatment of adenosine deaminase deficiency (Anderson 1998). Since then, more than 500 gene therapy protocols have been approved or implemented (Vorburger and Hunt 2002). In 2002, the successful gene-therapy-based cure for SCID was reported (Otsu and Candotti 2002). In 2003, the Chinese drug regulatory agency approved the first gene therapy product for head and neck squamous carcinoma under the trade name Gendicine (Zhaohui 2003). Currently, diseases with complex etiologies such as cancer (Galanis and Russell 2001; Mulherkar 2001) and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Baekelandt et al. 2000) are being targeted. In addition, DNA vaccines for malaria, AIDS, and many other diseases are in development (Bunnell and Morgan 1996). DNA vaccines have also been used to prevent allergic response (Horner et al. 2001).

2.2 *Oligonucleotides for Antisense and Antigene Applications*

Oligonucleotides are short single-stranded segments of DNA that upon cellular internalization can selectively inhibit the expression of a single protein. For antisense applications, oligonucleotides interact and form a duplex with the mRNA or the pre-mRNA and inhibit its translation or processing, consequently inhibiting protein biosynthesis. For antigene applications, oligonucleotides must enter the cell nucleus, form a triplex with the double-stranded genomic DNA, and inhibit the translation as well as the transcription process of the protein. On the molecular level, numerous mechanisms have been proposed to explain the basis of oligonucleotide action (Crooke 1999a, b; Speedie 2005). For therapeutic purposes, oligonucleotides can be used to selectively block the expression of proteins that are implicated in diseases (Akhtar et al. 2000). With successful antisense inhibition of proteins in animal models, the first antisense drug, fomivirsen sodium (Vitravene, Isis Pharmaceuticals, Carlsbad, CA), was approved for the treatment of cytomegalovirus retinitis in AIDS patients in 1998 (Crooke 1998b). Antisense oligonucleotides such as MG98 and ISIS 5132, designed to inhibit the biosynthesis of DNA methyltransferase and c-raf kinase respectively, are in human clinical trials for cancer (Mardan et al. 2002). Synthetic antisense DNA oligonucleotides and oligonucleotide analogs (Agarawal and Tang 1992), which inhibit the replication of several infectious agents such as hepatitis C virus (Alt et al. 1995), human cytomegalovirus (Azad et al. 1993), human immunodeficiency virus, and papilloma virus (Bordier et al. 1992; Cowser et al. 1993; Gerviax et al. 1997; Hanecak et al. 1996; Kinchington et al. 1992; Lisiewicz et al. 1992, 1993; Mirabelli et al. 1991; Morvan et al. 1993; Tonkinson and Stein 1993), have also been designed.

2.3 *Aptamers*

DNA aptamers are double-stranded nucleic acid segments that can directly interact with proteins (Stull and Szoka 1995). Aptamers interfere with the molecular functions of disease-implicated proteins or those that participate in the transcription or translation processes. Aptamers are preferred over antibodies in protein inhibition owing to their specificity, nonimmunogenicity, and stability of pharmaceutical formulation (Jayasena 1999). DNA aptamers have demonstrated promise in intervention of pathogenic protein biosynthesis against HIV-1 integrase enzyme (de Saultrait et al. 2002).

2.4 *DNAzymes*

DNAzymes are analogs of ribozymes with greater biological stability (Akhtar et al. 2000). The RNA backbone chemistry is replaced by the DNA motifs that confer improved biological stability. DNAzyme directed against vascular endothelial

growth factor receptor 2 was confirmed to be capable of tumor suppression by blocking angiogenesis upon intratumoral injections in mice (Zhang et al. 2002).

3 RNA-Based Therapeutics

3.1 RNA Aptamers

RNA aptamers are single-stranded nucleic acid segments that can directly interact with proteins (Stull and Szoka 1995). Aptamers recognize their targets on the basis of shape complementarity (Kaur and Roy 2008). Moreover, their binding specificity and affinity for the target are extremely high and similar to monoclonal antibodies. RNA aptamers have demonstrated promise in intervention of pathogenic protein biosynthesis against HIV-1 transcriptase (Chaloin et al. 2002). Moreover, RNA aptamers that specifically bind and inactivate vascular endothelial growth factor (VEGF) *in vitro* have been isolated. A clinical study on humans with injection of anti-VEGF aptamers in the eye showed that 80% of the patients retained or improved the eyesight, and they had no side effects (The Eyetech Study Group 2002).

3.2 RNA Decoys

The RNA decoys are designed to provide alternate, competing binding sites for proteins that act as translational activators or mRNA-stabilizing elements (Beelman and Parker 1995; Liebhauer 1997). Decoys can prevent translation or induce instability and, ultimately, destruction of the mRNA. Overexpressed short RNA molecules corresponding to critical *cis*-acting regulatory elements can be used as decoys for *trans*-activating proteins, thus preventing binding of these *trans* activators to their corresponding *cis*-acting elements in the viral genome (Sullenger et al. 1990, 1991). RNA decoys have an advantage over other nucleic acid-based strategies, that is, the decoys are less likely to be affected by variability of the infectious agent because any mutation in the *trans*-activating protein affects not only binding to the decoys but also binding to the endogenous targets. However, there is some question whether RNA decoy strategies will be as benign to the cell physiology as antisense RNAs, since it has been postulated that cellular factors may associate with them (Sullenger et al. 1990). It has previously been demonstrated that a cellular factor termed loop-binding protein is an absolute requirement for Tat-mediated trans-activation in an HIV-infected cell (Marciniak et al. 1990). Finally, RNA decoys do not function by sequestering either the Tat or Rev-protein but by sequestering the cellular factors such as the loop-binding protein.

3.3 *Antisense RNA*

Antisense drugs are short stretches of deoxyribonucleotide analogs that bind to specific complementary areas of the mRNA by Watson–Crick base pairing to block gene expression in a sequence-specific fashion. Antisense drugs may induce an RNaseH, which cleaves the mRNA at the site of binding, or can physically block translation or other steps in mRNA processing and transport to protein synthesis. The antisense drugs work at an early stage in the production of a disease-causing protein and theoretically can be applied to a number of diseases where the basic pathophysiology involves an overexpression of a given protein molecule (Crook 1998a, Speedie 2005).

Antisense ODNs can base-pair with a gene's transcript and constitute a new technology for the control of gene expression in prokaryotes and eukaryotes, including mammalian cells (Zamecnik and Stephenson 1978). The existence of naturally occurring RNAs and their role in regulating gene expression were shown in the mid-1980s (Simons and Kleckner 1983; Mizuno et al. 1984). Newer antisense oligonucleotides may offer improved pharmacokinetic and safety profiles because of reduced nonspecific interactions (Agarwal and Kandimalla 2000).

A stoichiometric disadvantage of antisense RNA is the high expression required to successfully bind to all target RNA. A major advantage is the lack of immunogenicity of antisense constructs, such that the oligonucleotides and the cells producing them will not be destroyed by the host immune response. The antisense approach to modulating gene expression has been extensively reviewed (Gewirtz et al. 1998; Scanlon et al. 1995; Stein 1998).

3.4 *Ribozymes*

Antisense RNA alone is not potent enough to produce complete inhibition *in vivo*. An enzymatic moiety can be included with antisense oligonucleotide, which will cleave the target RNA once the RNA–RNA duplex has formed. These enzymatic RNA strands are called “Ribozymes.” They are antisense RNA molecules that are capable of sequence-specific cleaving of RNA molecules (Stull and Szoka 1995). They function by binding to the target moiety through antisense sequence-specific hybridization and inactivating it by cleaving the phosphodiester backbone at a specific site. Thus, they can selectively bind to target mRNAs and form a duplex having highly distorted confirmation that is easily hydrolyzed, and this hydrolysis of mRNA may be used for targeted suppression of specific gene (Mardan et al. 2002). Two types of ribozymes, the hammerhead and hairpin ribozymes (the names are derived from their theoretical secondary structures), have been extensively studied owing to their small size and rapid kinetics (Earnshaw and Gait 1997; Hampel 1998) and for therapeutic applications (Stull and Szoka 1995). Hammerhead ribozymes cleave RNA at the nucleotide sequence U–H (H = A, C, or U) by hydrolysis of a 3'–5' phosphodiester bond, while hairpin ribozymes utilize the

nucleotide sequence C–U–G as their cleavage site (Breaker and Joyce 1994; Burke 1996; Usman et al. 1996). The presence of the RNA backbone in ribozymes makes them easy targets for degradation by RNases, so these molecules are biologically unstable in vivo (Mardan et al. 2002). Ribozymes can be used for knockout gene therapy by targeting overexpressed oncogenes such as the human epidermal growth factor receptor type-2 gene implicated in breast cancer (Aigner et al. 2001) and human papilloma virus infection. The development of ribozymes that colocalize in the same subcellular compartment as their target may further increase their effectiveness (Sullenger and Cech 1993). A significant limitation of the use of ribozymes for gene therapy is that they are susceptible to RNases.

3.5 *Small Interfering RNAs*

RNA interference (RNAi) is a posttranscriptional mechanism of gene silencing through chromatin remodeling, inhibition of protein translation, or direct mRNA degradation, which is ubiquitous in eukaryotic cells (Caplen 2004; Dorsett and Tuschl 2004; Shankar et al. 2005). Small interfering RNAs (siRNAs) can be used for downregulation of disease-causing genes through RNA interference.

Typically, these are short double-stranded RNA segments with 21–23 nucleotides and are complementary to the mRNA sequence of the protein whose transcription is to be blocked. On administration, siRNA molecules are incorporated into RNA-induced silencing complexes (RISC), which bind to the mRNA of interest and stimulate mRNA degradation mechanisms, such as nuclease activity, that lead to silencing of the particular gene (Bertrand et al. 2002; McMinus and Sharp 2002; Scherr et al. 2003).

The structure and functions of RISC have yet to be completely elucidated (Kurreck 2003). Introduction of foreign double-stranded RNAs (dsRNA) can initiate a potent cascade of sequence-specific degradation of endogenous mRNAs that bear homology to the dsRNA trigger (Fire et al. 1998). When dsRNAs are introduced into the cytoplasm, they are processed by the RNase III enzyme called Dicer, which cleaves long dsRNAs into short 21–23 nucleotide duplexes that have symmetric 2–3 nucleotide 3' overhangs and 5' phosphate and 3' hydroxyl groups (Tuschl et al. 1999; Hamilton and Baulcombe 1999). Although it was initially believed that effective RNAi required almost complete sequence homology throughout the length of the mRNA, now it appears that as few as seven contiguous complementary base pairs can direct RNAi-mediated silencing (Jackson and Linsley 2004). The use of siRNA as a therapeutic agent is still in its infancy. siRNAs are being investigated to inhibit HIV (Martinez et al. 2002), hepatitis (Zamore and Aronin 2003), and influenza infection (Ge et al. 2003). Moreover, RNAi technology has been applied to silence the expression of dominant mutant oncogenes, gene amplification, translocations, and viral oncogenes in order to elucidate their function and their interaction with other genes in a number of critical cellular pathways. Since siRNAs do not integrate into the genome and they offer greater safety than plasmid molecules, it is possible to

deliver a cocktail of siRNAs targeting multiple disease-causing genes in a single delivery system to control complex diseases such as cancer where several genes are malfunctioning (Wong 2001).

3.6 *MicroRNA*

MicroRNAs (miRNAs) are a class of naturally occurring, small noncoding RNA molecules 21–25 nucleotides in length. These molecules are partially complementary to messenger RNA (mRNA) molecules, and their main function is downregulation of gene expression via translational repression, mRNA cleavage, and deadenylation. MicroRNAs were first described in 1993 (Lee et al. 1993), and the term microRNA was coined in 2001 (Ruvkun 2001). Numerous miRNAs have been identified in various organisms hitherto. The central online repository for miRNA nomenclature, sequence data, annotation, and target prediction is miRBase, hosted by the Sanger Institute. MicroRNAs are transcribed by RNA polymerase II called pri-miRNAs that complete with a 5' cap and poly-A tail (Lee et al. 2004). In the nucleus, pri-miRNAs are processed into pre-miRNAs by the microprocessor complex, which consists of the RNase III enzyme Drosha (Han et al. 2004) and the double-stranded RNA Pasha/DGCR8 (Denli et al. 2004). These pre-miRNAs are exported by the karyopherin exportin (Exp5) and Ran-GTP complex (Yi et al. 2003). Then Ran GTPase binds with Exp5 to form a nuclear heterotrimer with pre-miRNAs (Yi et al. 2003; Lund et al. 2004). These pre-miRNAs are additionally processed by the RNase III enzyme Dicer (Bernstein et al. 2001) to generate miRNA. Moreover, Dicer is also responsible for the initiation of the formation of RISC, which is responsible for the gene silencing observed due to miRNA expression and RNA interference (Hammond et al. 2000; Hammond 2005).

4 Gene Transfer Technologies

Gene transfer technologies can be classified into three general types: electrical techniques, mechanical transfection, and vector-assisted delivery systems.

4.1 *Mechanical and Electrical Techniques*

Strategies of introducing naked DNA into cells by mechanical and electrical techniques include microinjection, particle bombardment, the use of pressure, and electroporation. Microinjection is highly efficient since one cell at a time is targeted for DNA transfer, but it is time consuming. Ballistic transfer of gold microparticles may be performed using particle bombardment equipment such as the gene gun.

Electroporation is achieved using high-voltage electrical current to facilitate DNA transfer that results in high cell mortality and is not suitable for clinical use (McAlister et al. 2000; Luo and Saltzman 2000; Regnier et al. 2000; Huang and Viroonchatapan 1999).

4.2 Vector-Assisted Delivery Systems

Vector-assisted DNA/gene delivery systems can be classified into two types based on their origin: biological viral DNA delivery systems and chemical nonviral delivery systems.

4.2.1 Viral Delivery Systems

In Viral Delivery Systems, nonpathogenic attenuated viruses can be used as delivery systems for genes/DNA molecules, especially plasmids (Kamiya et al. 2001; Mah et al. 2002; Lotze and Kost 2002). These viral DNA-delivery vectors include both RNA and DNA viruses. The viruses used as gene therapy vectors can be classified into four types: *Retroviruses* (McTaggart and Al-Rubeai 2002), *Adenoviruses*, *Adeno-associated viruses* (Martin et al. 2002), and *Herpes simplex viruses*. Gene expression using viral vectors has been achieved in tissues such as kidney (Lien and Lie 2002), heart muscle (Chamberlain 2002), eye (Martin et al. 2002), and ovary (Wolf and Jenkins 2002). Moreover, gene therapy using viral systems has made considerable progress for the treatment of a wide range of diseases, such as muscular dystrophy (Chamberlain 2002), AIDS (Lever 1996), and cancer (Zhao et al. 2002). Viruses are used in more than 70% of human clinical gene therapy trials worldwide (Walther and Stein 2000). The only approved gene therapy treatment (Gendicine) delivers the transgene using a recombinant adenoviral vector (Galanis and Russell 2001). DNA delivery using viral vectors has been extensively reviewed (Mah et al. 2002; Lotze and Kost 2002; Hale and Green 2002).

The first-generation retroviral vectors were largely derived from oncoretroviruses, such as the *Moloney Murine Leukemia virus* (MMuLV), and were unable to transfer genes into nondividing cells (Roe et al. 1993; Lewis and Emerman 1994). This limited the potential for their application as a delivery system in gene therapy. The utilization of the lentivirus family of retroviruses has overcome this shortcoming. Lentiviruses, which include Human immunodeficiency virus type 1 (HIV-1), *Bovine immunodeficiency virus* (BIV), *Feline immunodeficiency virus* (FIV), and *Simian immunodeficiency virus* (SIV), are able to transfer genes to nondividing cells (Lewis and Emerman 1994; Naldini et al. 1996). Retroviral vectors used in gene therapy are replication deficient, such that they are unable to replicate in the host cell and can infect only one cell (Mann et al. 1983; Cone and Mulligan 1984). This characteristic, although essential for the safety of viral vectors in gene therapy, imposes restrictions on the amounts of virus that can safely

be administered (Kim et al. 1998; Sheridan et al. 2000). Retroviral-mediated delivery of therapeutic DNA has been widely used in clinical gene therapy protocols, including the treatment of cancers, such as melanoma (Fujji et al. 2000) and ovarian cancer (Tait et al. 1999), adenosine deaminase deficiency–severe combined immune deficiency (Bordignon et al. 1995; Onodera et al. 1998), and Gaucher's disease (Dunbar et al. 1998). Retroviral vectors are capable of transfecting high populations (45–95%) of primary human endothelial and smooth muscle cells, a class of cells that are generally extremely difficult to transfer (Garton et al. 2002).

Adenoviruses have been used to deliver therapeutic DNA to patients suffering from metastatic breast, ovarian, and melanoma cancers (Stewart et al. 1999; Alvarez et al. 2000a, b). Indeed, the severe immune response of the host contributes to the limited survival of the adenoviral DNA in the targeted cells and results in a transient expression of the therapeutic gene since the adenoviral DNA is lost over time (Byrnes et al. 1995; Yang et al. 1996a, b; van Ginkel et al. 1997; Michou et al. 1997). First-generation adenoviral vectors were able to accommodate the introduction of therapeutic genes over 7 kb long (but rarely larger) into targeted cells (Bett et al. 1993). However, the generation of gutless adenoviral vectors, which lack all viral genes, has facilitated adenoviral delivery of up to 30 kb of a therapeutic DNA sequence (Clemens et al. 1996; Kochanek et al. 1996; Parks and Graham 1997; Morsy et al. 1998) with decreased toxicity (Schiedner et al. 1998). Adenoviral-mediated gene transfer in COS-7 cells was significantly higher than that achieved by liposomal delivery systems (Heider et al. 2000).

The use of Adeno-Associated Viral (AAV) vector provides an alternative to adenoviral vectors for gene therapy and a means for long-term gene expression with a reduced risk of adverse reactions upon administration of the vector (Fisher et al. 1997; Jooss et al. 1998). AAV viruses are linear, single-stranded DNA parvoviruses that are not associated with any disease in humans (Rose et al. 1969). In humans, the site of AAV viral DNA integration is on chromosome 19 (Kotin et al. 1990; Samulski et al. 1991). In the engineering of AAV vectors, most of the AAV genome can be replaced with the therapeutic gene (Samulski et al. 1989), which significantly reduces potential adverse responses of the host to viral infection. However, the size of the therapeutic gene is limited to approximately 5 kb (Dong et al. 1996; Hermonat et al. 1997). First-generation adeno-associated viruses had a very small capacity of ~4.7 kb for encapsulation of the plasmid DNA cargo. Recent reports demonstrate efficient production of second-generation adeno-associated viruses with higher encapsulating capabilities (Owens 2002). It has been demonstrated that adenoviruses in formulations may lose their potency after storage in commonly used pharmaceutical vials (Nyberg-Hoffman and Aguilar-Cardova 1999). Herpes simplex virus (HSV) vector is a large and relatively complex enveloped, double-stranded DNA virus that has the capacity to encode large therapeutic genes and, like AAV, can remain latent in infected cells, providing the potential for long-term expression of the therapeutic gene (Carpenter and Stevens 1996). Although able to infect many cell types, HSV vectors currently are limited in their use by vector toxicity (Lowenstein et al. 1994).

4.2.2 Nonviral Delivery Systems

Nonviral delivery systems have the greatest advantage over viral delivery systems—the lack of immune response and ease of formulation and assembly. Commonly used nonviral vectors for delivery of DNA-based therapeutics can be classified into three major types: *Naked DNA delivery systems*, *polymeric delivery systems*, and *liposomal delivery systems* (Mardan et al. 2002; Fattal et al. 1999, 2001; Pedrosa de Lima et al. 2001).

Naked DNA can be administered via two possible routes, either by ex vivo delivery or by in vivo delivery. The ex vivo method of naked DNA delivery has been used successfully for the introduction of DNA into endothelial and smooth muscle cells (Nakamura et al. 1998; Mann et al. 1999a, b); its reliance on the culture of harvested cells renders it unsuitable for many cell types. In vivo delivery of naked DNA was first described in 1990 (Wolff et al. 1990). Efficiency of the delivery of naked DNA can be improved when administered in a pressure-mediated fashion (Mann et al. 1999a, b; Liu et al. 1999). Particle bombardment technology enables the localized delivery of DNA readily into skin or muscle (Fynan et al. 1993). Another technique for delivery of naked DNA directly into target cells is electroporation. The successful delivery of DNA by electroporation in vivo has been reported in tissues such as skin and muscle (Wong and Neumann 1982; Neumann et al. 1982; Rols et al. 1998; Rizzuto et al. 1999).

In polymeric delivery systems, cationic polymers are used in gene delivery because they can easily complex with the anionic DNA molecules (Hwang and Davis 2001). The mechanism of action of these polycomplexes is based on the generation of a positively charged complex owing to electrostatic interaction of these cationic polymers with anionic DNA (Luo and Saltzman 2000). Commonly used polymers include polyethylenimine (Lemkine and Demeneix 2001), poly-L-lysine (Lollo et al. 2002), chitosans (Borchard 2001), and dendrimers (Mardan et al. 2002). Agents such as folates, transferrin, antibodies, or sugars such as galactose and mannose can be incorporated for tissue targeting (Mardan et al. 2002). Synthetic polymers such as protective interactive noncondensing polymers (PINC), poly-L-lysine, cationic polymers, and dendrimers offer an alternative to cationic lipids as a vehicle for DNA delivery into target cells (Boussif et al. 1995; Wadhwa et al. 1995; Kukowska-Latello et al. 1996; Tang and Szoka 1997; Mumper et al. 1998). Encapsulation of a DNA molecule or even a therapeutic viral vector within a biodegradable polymer has been demonstrated to permit the controlled release of the DNA in a targeted cell over a period of weeks or months (Naughton et al. 1992; Singh et al. 2000). The inclusion of proteins and peptides in the DNA complex, which are recognized by receptors on targeted cells, has led to an improvement in the efficiency of DNA uptake in several instances (Jenkins et al. 2000). Some polymers have inherent potent pharmacological properties (such as hypercholesterolemia induced by chitosans) that make them extremely unfavorable for human use (LeHoux and Grondin 1993).

Liposomes are one of the most versatile tools for the delivery of DNA therapeutics (Akhtar et al. 2000; Fattal et al. 1999, 2001; Godbey and Mikos 2001). Liposome

and drug/lipid complexes have been used for the delivery of the anticancer drugs doxorubicin and daunorubicin (Tejada-Berges et al. 2002). Liposomes can be used as DNA drug delivery systems either by entrapping the DNA-based therapeutics inside the aqueous core or complexing them to the phospholipids lamellae. Liposome can also be used for specialized gene delivery options, such as long circulation half-life and sustained and targeted delivery (Fattal et al. 1999). Numerous studies have demonstrated the use of cationic liposomal formulations for the delivery of different plasmid constructs in a wide range of cells, both in vivo and in vitro (Marshall et al. 1999). The use of cationic lipids to transfer DNA into cells was first described as an in vitro method of DNA delivery (Felgner et al. 1987). Cationic liposomes have also been used in clinical trials to deliver therapeutic DNA (Caplen et al. 1995; Hyde et al. 2000; Noone et al. 2000; Nabel et al. 1993, 1996). Cationic liposomal formulations consist of mixtures of cationic and zwitterionic lipids (Godbey and Mikos 2001; Felgner et al. 1994; Hofland et al. 1996). Proprietary formulations of cationic lipids such as Lipofectamine (Invitrogen, Carlsbad, CA), Effectene (Qiagen, Valencia, CA), and Transfectam (Promega, Madison, WI) are commercially available (Kang et al. 1999), but most of the kits are useful only for in vitro experimentation. There are reports of improved efficiency of DNA delivery by cationic lipid via the coupling of specific receptor ligands or peptides to DNA/liposome complexes (Jenkins et al. 2000; Ellison et al. 1996; Simoes et al. 1998; Shinmura et al. 2000; Compton et al. 2000). Cytotoxicity of cationic lipids has been established in numerous in vitro (Lappalainen et al. 1994; Patil et al. 2004) and in vivo (Dokka et al. 2000; Fillion and Philips 1997; Freimark et al. 1998) studies. Low transfection efficiencies have been attributed to the heterogeneity and instability of cationic lipoplexes (Lee et al. 2001). Another drawback in the use of cationic lipids is their rapid inactivation in the presence of serum (Hofland et al. 1996; Audouy et al. 2000). Some in vivo studies have revealed that the gene transduction responses obtained by cationic liposomes were transient and short-lived (Liu et al. 1997; Wheeler et al. 1996). As an alternative to cationic lipids, the potential of anionic lipids for DNA delivery has been investigated. The safety of anionic lipids has been demonstrated when administered to epithelial lung tissue. In recent years, a few studies using anionic liposomal DNA delivery vectors have been reported. There have been attempts to incorporate anionic liposomes into polymeric delivery systems. However, these vectors have limited applications, mainly because of (1) inefficient entrapment of DNA molecules within anionic liposomes and (2) lack of toxicity data. Lack of further progress of these systems may be attributed, in part, to the poor association between DNA molecules and anionic lipids, caused by electrostatic repulsion between these negatively charged species (Patil et al. 2004, 2005; Dokka et al. 2000; Patil and Rhodes 2000a, b; Fillion et al. 2001; Lakkaraju et al. 2001; Lee and Huang 1997; Guo et al. 2002; Perrie and Gregoriadis 2000). Along with numerous cationic and anionic lipid derivatives, functionalized liposomal formulations serving specific therapeutic objectives have shown promise in gene therapy (Fattal et al. 1999; Venugopalan et al. 2002; Maclean et al. 1997). Specialized liposomal delivery platforms include pH-sensitive liposomes, immunoliposomes, and stealth liposomes. pH-sensitive liposomes can be generated

by the inclusion of 1,2-dioleoyl-3-phosphoethanolamine (DOPE) into liposomes composed of acidic lipids such as cholesterylhemisuccinate or oleic acid. At the neutral cellular pH 7, these lipids have the typical bilayer structure; however, upon endosomal compartmentalization, they undergo protonation and collapse into a nonbilayer structure, thereby leading to the disruption and destabilization of the endosomal bilayer, which in turn helps in the rapid release of DNA into the cytoplasm (Venugopalan et al. 2002). Efficient gene delivery of the beta-galactosidase and luciferase reporter plasmids has been obtained using pH-sensitive liposomes in a variety of mammalian cell lines (Legendre and Szoka 1992). A chemical derivative of DOPE, cCitraconyl-DOPE, has been used to deliver DNA-based therapeutics to cancer cells, thereby combining the targeting and the rapid endosome-releasing aspects of specialized liposomal delivery systems (Reddy and Low 2000). A phosphatidylcholine/glycyrrhizin combination was also successful in pH-sensitive gene delivery in mice (Sviridov et al. 2001). Immunoliposomes are sophisticated gene delivery systems that can be used for cell targeting by the incorporation of functionalized antibodies attached to lipid bilayers (Maclean et al. 1997). Immunoliposomes containing an antibody fragment against the human transferrin receptor were successfully used in targeted delivery of tumor-suppressing genes into tumors in vivo (Xu et al. 2002). Tissue-specific gene delivery using immunoliposomes has been achieved in the brain (Shi et al. 2001), embryonic tissue (Khaw et al. 2001), and breast cancer tissue (Krauss et al. 2000). Stealth liposomes are sterically stabilized liposomal formulations that include polyethylene glycol (PEG)-conjugated lipids (Fattal et al. 1999).

Recently, polyethylenimine (PEI), poly(lactic-co-glycolic acid) (PLGA), polypeptides, chitosan, cyclodextrin, dendrimers, and polymers containing different nanoparticles are used in vitro and in vivo with respect to their structure, physicochemical properties, and delivery efficiency as an siRNA delivery vehicle (Kaushik et al. 2011).

5 Current Status of Gene Therapy Research

The FDA has not yet approved any human gene therapy product for sale. Current gene therapy is experimental and has not proven very successful in clinical trials. Little progress has been made since the first gene therapy clinical trial began in 1990. In 1999, gene therapy suffered a major setback with the death of 18-year-old Jesse Gelsinger.

Another major blow came in January 2003 when the FDA placed a temporary halt on all gene therapy trials using retroviral vectors in blood stem cells. FDA took this action after it learned that a second child treated in a French gene therapy trial had developed a leukemia-like condition. Both that child and another who had developed a similar condition in August 2002 had been successfully treated by gene therapy for X-linked severe combined immunodeficiency disease (X-SCID), also known as “bubble baby syndrome.”

FDA's Biological Response Modifiers Advisory Committee (BRMAC) met at the end of February 2003 to discuss possible measures that could allow a number of retroviral gene therapy trials for treatment of life-threatening diseases to proceed with appropriate safeguards.

In April of 2003, the FDA eased the ban on gene therapy trials using retroviral vectors in blood stem cells.

6 Recent Developments in Gene Therapy

Month/year	Development in gene therapy	Author(s) name; name of journal(s)/URL	Title of the article	Summary/inference
March 2009	Nanotechnology and gene therapy yields treatment to torpedo cancer	Schatzlein A [http://news.bbc.co.uk/2/hi/health/7935592.stm/ (March 10)]	Nano-treatment to torpedo cancer	School of Pharmacy in London is testing a treatment in mice, which delivers genes wrapped in nanoparticles to cancer cells
April 2008 April 2008	Results of world's first gene therapy for inherited blindness show sight improvement	1. James et al.; N Engl J Med 2. Albert et al.; N Engl J Med	1. Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis 2. Safety and Efficacy of Gene Transfer for Leber's Congenital Amaurosis	UK researchers from the UCL Institute of Ophthalmology and Moorefield's Eye Hospital NIHR Biomedical Research Centre have announced results from the world's first clinical trial to test a revolutionary gene therapy treatment for a type of inherited blindness (Leber's congenital amaurosis). The results show that the experimental treatment is safe and can improve sight. The findings are a landmark for gene therapy and could have a significant impact on future treatments for eye diseases
May 2007	This is the first trial to use gene therapy in an operation to treat blindness in humans	Ben H [http://www.reuters.com/article/scienceNews/idUSL016653620070501?/ (May 1, 2007)]	Doctors test gene therapy to treat blindness	A team of British doctors from Moorefield's Eye Hospital and University College in London conduct the first human gene therapy trials to treat Leber's congenital amaurosis, a type of

(continued)

Month/year	Development in gene therapy	Author(s) name; name of journal(s)/URL	Title of the article	Summary/inference
				inherited childhood blindness caused by a single abnormal gene. The procedure has already been successful at restoring vision for dogs
Jan 2007	A combination of two tumor-suppressing genes delivered in lipid-based nanoparticles	Scott M [http://www.newswise.com/p/articles/view/526526/] (January 11, 2007)]	Dual gene therapy suppresses lung cancer in preclinical test	A combination of two tumor-suppressing genes delivered in nanoparticles drastically reduces the number and size of human lung cancer tumors in mice during trials conducted in the University of Texas
Aug 2006	This is the first time that gene therapy is used to successfully treat cancer in humans	Morgan et al. [http://www.cancer.gov/newscenter/pressreleases/MelanomaGeneTherapy/] (August 31, 2006)]	Cancer regression in patients mediated by transfer of genetically engineered lymphocytes	Researchers at the National Cancer Institute (NCI) successfully reengineer immune cells, called lymphocytes, to target and attack cancer cells in patients with advanced metastatic melanoma
March 2006	This is the first study to show that gene therapy can cure diseases of the myeloid system	[http://www.cincinnatichildrens.org/] (March 31, 2006)]	Gene therapy appears to cure myeloid blood diseases in ground-breaking international study	Gene therapy is effectively used to treat two adult patients for a disease affecting nonlymphocytic white blood cells called myeloid cells. Myeloid disorders are common and include a variety of bone marrow failure syndromes
March 2003	This method has potential for treating Parkinson's disease	Anil A [http://www.newscientist.com/section/science-news/] (March 22, 2003)]	Undercover genes slip into the brain	University of California research team gets genes into the brain using liposomes coated in polyethylene glycol. The transfer of genes into the brain is a significant achievement because viral vectors are too big to get across the "blood-brain barrier"

(continued)

Month/year	Development in gene therapy	Author(s) name; name of journal(s)/URL	Title of the article	Summary/inference
March 2003	RNA interference or gene silencing may be a new way to treat Huntington's	Bob H [http://www.newsscientist.com/section/science-news/ (March 13, 2003)]	Gene therapy may switch off Huntington's	Short pieces of double-stranded RNA (siRNAs) are used by cells to degrade RNA of a particular sequence. If an siRNA is designed to match the RNA copied from a faulty gene, then the abnormal protein product of that gene will not be produced
Oct 2002	Technique has potential to treat the blood disorder thalassemia, cystic fibrosis, and some cancers	Danny P [http://www.newsscientist.com/section/science-news/ (October 11, 2002)]	Subtle gene therapy tackles blood disorder	New gene therapy approach repairs errors in messenger RNA derived from defective genes
Oct 2002	Gene therapy for treating children with X-SCID	Emma Y [http://www.newsscientist.com/section/science-news/ (October 3, 2002)]	"Miracle" gene therapy trial halted	Gene therapy for treating children with X-SCID (severe combined immunodeficiency) is stopped in France when the treatment causes leukemia in one of the patients
May 2002	Researchers are able to create tiny liposomes for transferring therapeutic DNA	Sylvia PW [http://www.newsscientist.com/section/science-news/ (May 12, 2002)]	DNA nanoballs boost gene therapy	Researchers at Case Western Reserve University and Copernicus Therapeutics are able to create tiny liposomes that can carry therapeutic DNA through pores in the nuclear membrane
March 2002	Sickle cell is successfully treated in mice	Jennifer FW; The Scientist 16:36 [http://www.the-scientist.com/2002/3/18]	Murine Gene Therapy Corrects Symptoms of Sickle Cell Disease	The scientists corrected the murine symptoms of this chronic, inherited, and often painful disorder (sickle cell disease)

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From Nucleic Acids Sequences to Molecular Medicine

Erdmann (Deceased), V.A.; Barciszewski, J. (Eds.)

2012, X, 654 p., Hardcover

ISBN: 978-3-642-27425-1