11 DRUG TARGETING

Drugs become effective when it reaches the target site in the body after administration. Before or after reaching the target site, however, drugs reache other places in the body too. In fact, drugs are distributed throughout the body and this causes undesirable side effects. It would be most desirable if a drug can be delivered only to specific organs and tissues so that occurrences or severity of systemic adverse effects are reduced. Thus, an ideal drug-delivery system require the ability to find the specific target. Furthermore, drug delivery only at the target site will reduce the total amount of drug necessary for the treatment of diseases. Current technology is not advanced enough to design a "magic bullet" drug-delivery system yet, but significant advances have been made in drug targeting.

I. DRUG TARGETING BY LOCALIZED DELIVERY

A. TARGETING TO SURROUNDING TISSUES FROM IMPLANTED SITES

1. Local Delivery from Stents

The most likely modes of drug delivery, including genes, to cardiovascular tissues will be catheters, stents on catheters, direct injection into the myocardium and/or pericardium during surgery, and direct injection into peripheral skeletal muscle (for peripheral angiogenesis). These modes of local delivery present a major advantage for cardiovascular gene therapy (compared to other organ systems). A stent is a tubular-shaped, expandable steel scaffold that can be delivered to the site of a closure or blockage by a balloon catheter, then expanded to the full diameter of the artery.

When a coronary vessel is blocked, a balloon catheter is placed at the site and the atherosclerotic lesion is cleared by inflating the balloon. To maintain the open vessel, a metal stent is deployed. Usually, muscle cells grow and proliferate to form neointima and block the vessel. This process is known as restenosis. Stents have become a very useful tool for the treatment of restenosis, and is estimated that approximately 40% of primary angioplasties in the United States are now being performed with stents. The recurrence of the blockage can be delayed or even prevented by delivering various drugs including paclitaxel and antisense oligonucleotides and ribozyme (ribonucleic acid enzyme or catalytic RNA) from the stent. Since the drug is released locally to the target tissue, the amount of drug required can be much smaller than that required by system delivery.

A number of clinical studies showing the highly beneficial effect of localized drug delivery from stents were reported at the American Heart Association's Scientific Sessions 2001 Meeting in Anaheim, CA. The in-stent restenosis rate in the patient group with 2.7 μg/mm^2 of paclitaxel was only 3% as compared with more than 20% of the control group. It is no longer a question whether the localized drug delivery from the stent is beneficial or not. The question now is what drug should be released at what rate for how long? This is just the beginning of the controlled drug delivery from the stents.

The Conor stent specifically designed for localized drug delivery.

The holes of the Conor stents can be filled with drugs. The same drug can be loaded at different concentrations in different holes and/or different drugs can be loaded in different places of the same stent.

B. Drug Activation at the Target Site

Photodynamic therapy, which uses tiny light emitting diodes (LEDs) to activate light-sensitive, tumor-treating drugs, was successfully used in surgery to treat brain cancer (Roy, 1999). In this technique, a drug called Photofrin® is injected into the patient's bloodstream. Photofrin® is a light-activated drug currently approved in the U.S. for treating certain lung and

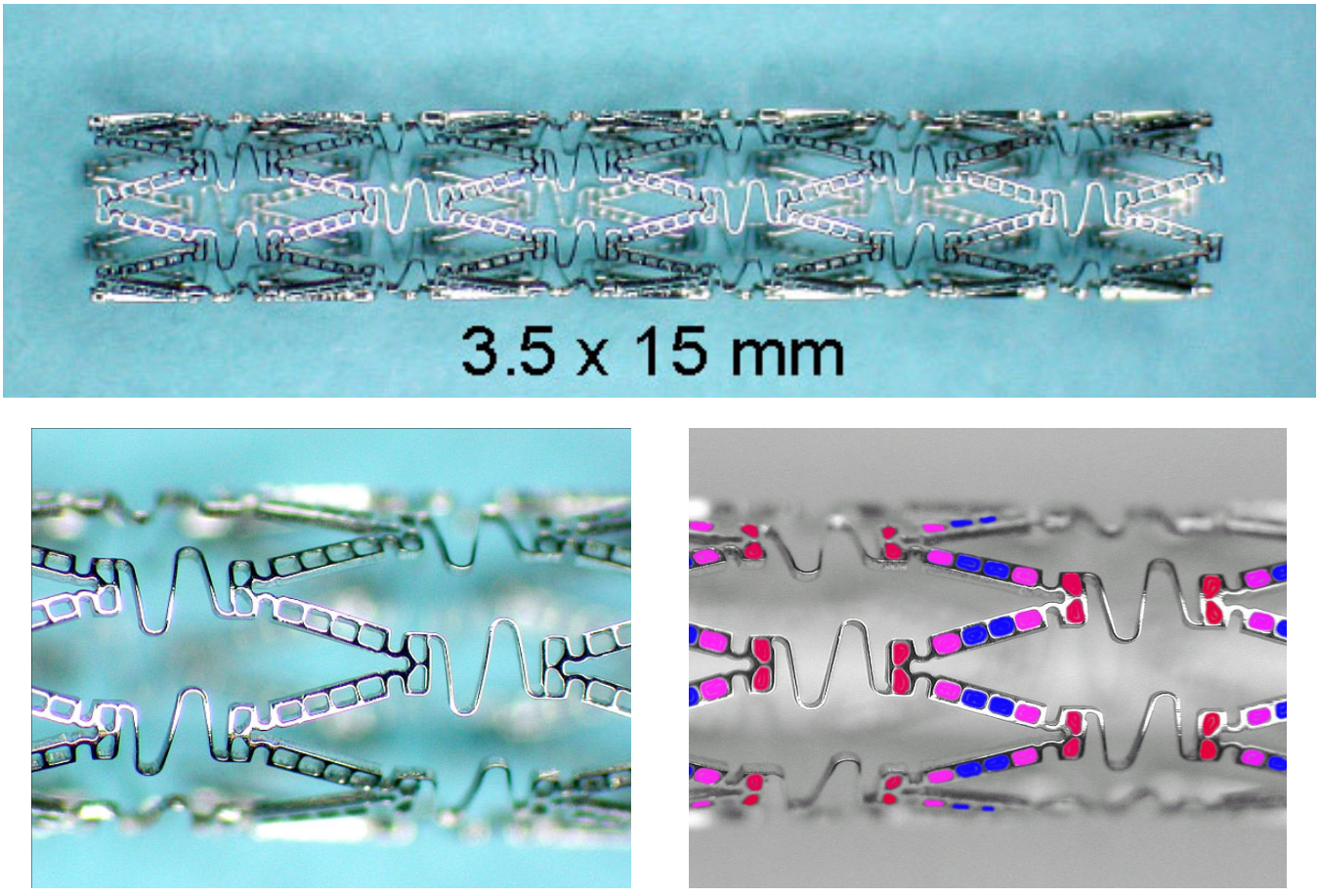

Figure 11.1 Photomicrographs of Conor stents.

esophageal cancers. Then, LED probe is placed near the affected tissue to illuminate the tumor and activate Photofrin®. The photodynamic therapy was originally developed for plant-growth research aboard NASA's space shuttle. The LEDs long, cool wavelengths of light illuminates through all nearby tissues, reaching parts of the tumor that the shorter wavelengths laser light cannot. When special tumor-fighting drugs are illuminated with LEDs, the tumors are more effectively destroyed than with conventional surgery.

II. TARGETING IN BLOOD CIRCULATION

A. ACTIVE TARGETING (TARGETING WITH HOMING DEVICES)

1. Drug-Homing Device Conjugates

The most widely used homing device for drug targeting has been antibodies. The premise of antibody-mediated targeting is that immunconjugates (*i.e.*, antibody-drug) can find their way to the target site owing to the specific antigen-antibody interactions. To increase the efficiency of drug delivery, the antibody can be linked to a polymer chain with numerous drug molecules. Such antibody–drug–polymer conjugates can release drugs by enzymatic or chemical degradation as described in Chapter 9. Targeting with monoclonal antibodies became possible with the work by Köhler and Milstein (Köhler & Milstein, 1975) that allows production of unlimited quantities of monoclonal antibodies directed against specific cell surface

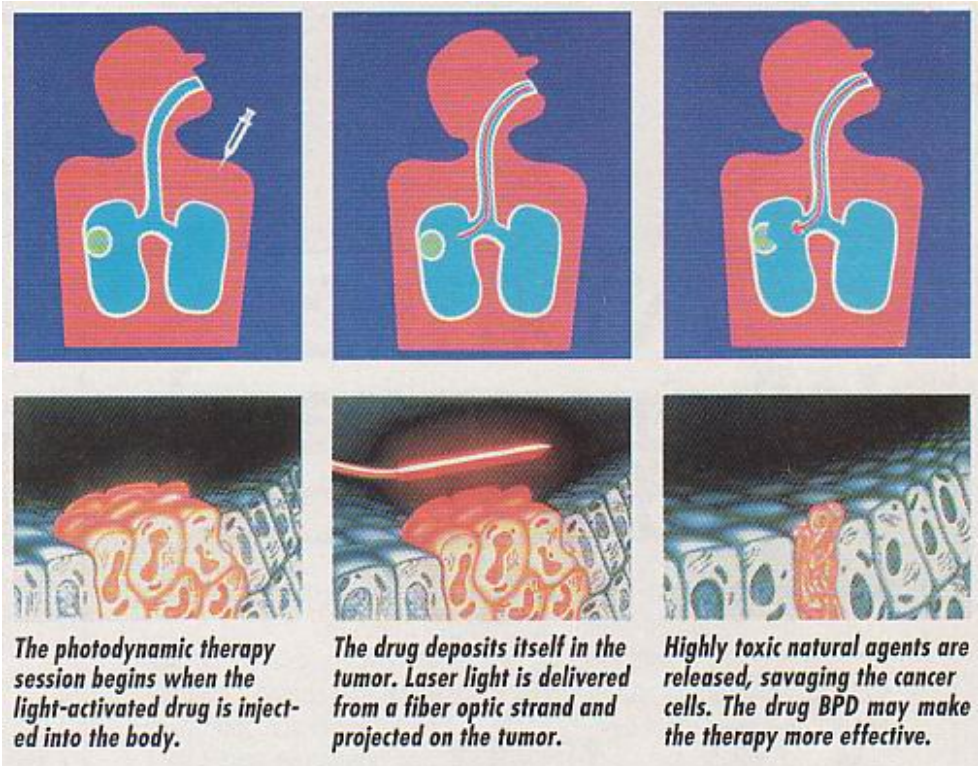

Figure 11.2 Schematic of the light activation of Photofrin® (Mallozzi, 1995).

receptors (*e.g.*, cell surface determinants) present on tumor cells. Monoclonal antibodies are cytotoxic themselves and can be used to kill cells, but the monoclonal antibodies linked to drugs and toxins are even more effective.

In addition to antigen–antibody interaction, specificity to target molecules can also be achieved by a variety of molecules, such as lectins, viruses, immunotoxins, and carbohydrates. These molecules are called "homing devices." The active targeting that utilizes homing devices is highly promising in theory, although all its potentials have not realized in practice. For this approach to be successful, it is essential to select a homing device against the ligand or receptor that is rather abundant at the target site.

The use of antibodies as a homing device should be taken with caution. Many patients who received infusions of the early monoclonal therapeutic antibodies developed their own antibodies against the monoclonal antibodies, which are foreign to the body, making patients even sicker (Ezzell, 2001). The patients experienced a so-called HAMA (human anti-mouse antibodies) response, resulting in joint swelling, rashes, and kidney failure. Furthermore, it was shown that the antibodies were sopped up in the liver before reaching the target cells. The failure of the early clinical applications of monoclonal antibodies was mainly due to the use of murine antibodies that are perceived by the human immune system as interlopers.

To avoid the HAMA response and premature destruction of mouse antibodies, a number of new approaches have been developed. The first approach is production of chimeric (part mouse, part human) antibodies. About two thirds of the antibody is made of human components. An example is ReoPro® (Centocor: Malvern, PA) for prevention of blood clot by binding to platelet receptors. The body is known not to make antibodies targeted to healthy tissues. If it does, it is called autoimmune disease. The second strategy is called humanization. Herceptin from Genentech is an example. In humanization of antibodies, genetic engineering is used to selectively replace as much of the murine antibodies as possible with human components. About 90% of the produced antibodies are made of human components. Only specific antigen-binding sites are from mouse. The third method is to generate fully human antibodies by fusing human B cells into immortalized cells to create hybridomas. The technologies of humanization and making fully human antibodies have made the monoclonals more commercially viable than ever before.

2. Improvements to be made in Targeting to Cancer Cells

Targeted therapy has been extensively studied for cancer therapy because chemotherapy is usually terminated by toxic effects on normal cells. To date, *in vivo* results of antibody-mediated treatment of cancer drugs, such as doxorubicin, daunorubicin, and vindesine, have been rather disappointing. Improvement in the *in vivo* results by the antibody-mediated targeting requires overcoming many technical problems listed in Table 11.1.

Table 11.1 Technical Challenges in Antibody-Mediated Targeting

Preservation of the antibody functions after conjugation.

Increase in the stability of the chemical linkages.
Increase in the possibility of antibody binding to the target molecule on the cell surface.
Increase in endocytosis.
Reduction in repeated treatment.

Antibodies may undergo conformational changes after conjugation, and thus may reduce their ability to bind to cell surface receptors. *In vivo* stability of the chemical linkages of immunoconjugates may not be adequate to preserve drug and antibody activity. Since receptor molecules are shed into the plasma, the shed receptor molecules may bind to monoclonal antibodies before the conjugate has a chance to reach its target. Once the conjugates reach the target cells, they should have the ability to cross through the cell membranes. It is also important to increase the penetration of the immunoconjugates into the center of the solid tumors. Since human monoclonal antibodies are difficult to obtain, repeated treatments of non-human antibodies may cause immunogenic reactions.

Frustration of monoclonal antibody research mainly results from unreasonably high expectations of the magic-bullet dream. There are many instances that show successful treatment of cancers using monoclonal antibodies. In cases where tumor cells have abundant antigens on the surface and allows rapid endocytosis of the bound conjugates, antibody-mediated targeting showed very encouraging results. Antibody BR96-doxorubicin conjugates effectively cured human lung adenocarcinoma, colon carcinoma, and breast carcinoma growing as subcutaneous transplants in athymic mice (Trail *et al*., 1993). The tumor cells have more than 200,000 antigen molecules pre cell and rapidly endocytosed the conjugates after binding to the cell receptors. In cases where tumor cells do not shed antibodies, the monoclonal treatment also are highly successful. Non-Hodgkin's lymphomas (which are cancers of the body's antibody-producing immune system cells, particularly the B-cells) afflict some 225,000 Americans including Jacqueline Onassis, with over 50,000 new cases expected every year. The antibody targets a protein (the CD20 antigen) expressed on the surface of mature B cells and on B-cell tumors but not on B-cell precursors or other body tissues. In addition, the antibody does not shed from the surface of the cell, nor does it internalize. The antibody works by binding to its target antigen and recruiting the patient's natural defenses to attack and kill both malignant and normal mature B cells. Trials to date show that normal B cells regenerate (from stem cells) and return to normal levels within months after treatment. The overall response rate was 50%, which is remarkable for any one drug in managing the fatal immune system cancer.

3. Blocking of the Blood Vessels Leading to Tumors

Since tumors must have an adequate blood supply to sustain their growth, many strategies have been used to interrupt the blood supply to the tumors. One of them is to induce blood clotting at the site of tumors. The formation of blood clots (thrombosis) within the tumor vessels was tested as a therapy for solid tumors in a mouse model (Huang *et al*., 1997).

Thrombosis was initiated by targeting the cell surface domain of human tissue factor, using a bispecific antibody, to an experimentally induced marker on tumor vascular endothelial cells. The unique approach here is the use of the truncated form of tissue factor (tTF). It had limited ability to initiate thrombosis when free in the circulation, but became an effective and selective thrombogen when targeted to tumor endothelial cells (Huang *et al*., 1997). Complete tumor regressions was observed in 38% of the tested mice with large neuroblastomas after IV administration of the antibody-tTF complex.

An alternative to the clotting factors, swelling polymers (such as hydrogels with high-swelling ratios) can be used to block the blood vessels by injecting them near the tumor sites.

The approach of selective occlusion of the vasculature of solid tumors using immunoconjugates or embolizing agents presents several advantages over direct targeting of tumor cells (Huang *et al*., 1997). First, local interruption of a blood supply to the tumor will lead to an avalanche of tumor cell death. Second, the tumor vascular endothelium is directly accessible by immunoconjuagates, while the tumor cells are poorly accessible owing to their location in outside the bloodstream.

4. Antiangiogenesis

Recently, a new wave of potential cancer therapies are being developed. Instead of direct attack on cancer cells with chemotherapeutic agents, the blood vessel that supplies oxygen and nutrients to tumor cells can be shut off. Since tumors requires growth of new blood vessels, blocking this process, called angiogenesis, became a useful anticancer strategy. Many agents have been identified that interfere with the endothelial cells essential in building the new vessels. Antiangiogenic agents work either by preventing endothelial cells from responding to growth factors or by suppressing their ability to chew their way through surrounding tissues. An effective vessel-targeting therapy would be useful for various types of cancer since the approach is not tumor-specific. In addition, the approach targets normal endothelial cells in tumor vasculature instead of tumor cells. This makes it unlikely that tumor cells could mutate to render them resistant to therapy.

Judah Folkman at Harvard Medical School, who is the pioneer in the antiangiogenic approach, discovered a number of antiangiogenic agents, such as platelet factor 4 (PF-4) made by blood platelets, fumagillin produced by molds, and endostatin (one of the most potent in a growing collection of molecules that block new blood vessel formation). The work done at Genentech, Inc. showed that antibodies arresting the activity of an angiogenic protein called vascular endothelial growth factor (VEGF) slowed the growth of several types of tumors in mice. Since then, efforts have been directed toward developing drugs that block the action of VEGF or basic fibroblast growth factor, both of which stimulate angiogenesis by enhancing endothelial cell growth. Another potential target for growth-factor inhibitors is a new family of receptors, called TIE receptors (for tyrosine kinase with immunoglobulin- and EGF-like domains) that

exist almost exclusively on endothelial cells. Angiopoietins are a new family of naturally occurring molecules that either activate or inhibit the TIE receptors, which in turn influence endothelial cell growth (Barinaga, 1996). Other drugs that show antiangiogenic activity are thalidomide, interleukin-12 (IL-12), metalloproteinase inhibitors, and integrin inhibitors. Metalloproteinase inhibitors block enzymes secreted by cancer cells that break down proteins of the extracellular matrix enabling the cells to slip through the surrounding tissue and spread. Integrin v3, found on the surface of endothelial cells, interact with the proteins of the extracellular matrix to help the cells differentiate, migrate, and divide for forming new blood vessels. The successful treatment of tumors using antiangiogenic agents requires targeting of the agents to the tumor sites.

B. PASSIVE AND PHYSICAL TARGETING

Drug-delivery systems in the form of nanoparticles, microparticles, or liposomes, have been used for drug targeting upon intravenous (IV) injection. These particulate systems may possess homing devices at their surfaces. But, quite often they can reach the target sites without homing devices.

1. Passive Targeting by Particle Size

Most of the injected microparticulate drug-delivery systems are rapidly cleared from the systemic circulation by the reticuloendothelial system (RES). RES consists of fixed macrophages of the liver (Kupffer cells), spleen, lungs, and bone marrow as well as circulating monocytes. RES is also called mononuclear phagocytic system (MPS). Particles larger than 10 μm are known to be completely trapped in the lung capillaries by mechanical filtration. On the other hand, particles smaller than about 2 μm are rapidly captured by the macrophages of RES and deposited mainly in the liver and spleen. Thus, if the target site is lung, liver, or spleen, targeting can be achieved rather easily. This is called the passive targeting.

Uptake by the RES can be reduced to a certain extent by coating the surface of particles with hydrophilic polymers such as poly(ethylene oxide) (PEO) or PEO-containing block copolymers (*e.g.*, Poloxamers (Pluronics®) and Poloxamines (Tetronics®)). This is the same approach as the PEGylation method used to extend the blood circulation time for protein drugs.

Passive targeting still allows targeting of microparticles to certain tumor sites. Tumors attract new blood vessels to receive nutrients by a process called angiogenesis. The new blood vessels leading to tumors are usually leakier than the normal blood vessels. This particular property makes preferential delivery of microparticles to tumor sites possible.

2. Physical Targeting

Particulate drug-delivery systems can be made to release more drugs upon small changes in environmental condition, such as pH and temperature. Small pH changes that may exist between the target site and surrounding tissues can be used to release drugs at a selected site. Local temperature

can be altered by applying heat pad or ice pad around the target site. Electrical and magnetic field can be used to capture the particulate drug delivery systems at target site or close-vicinity of the target.

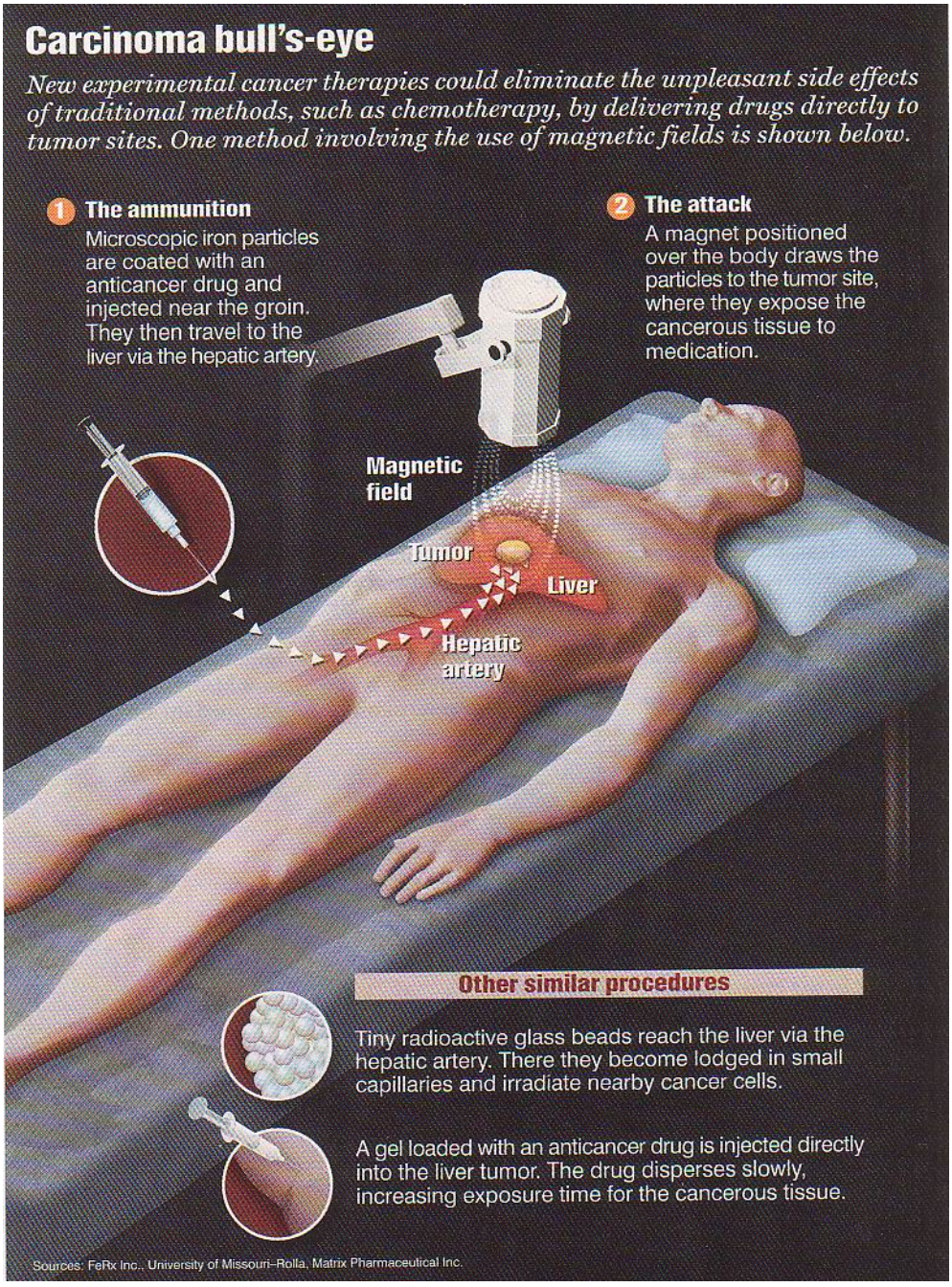

Figure 11.3 Illustration of physical targeting (Sobel, 2000).

III. TARGETING IN THE GI TRACT

A. TARGETING TO STOMACH

Targeting of drug-delivery systems to the specific region of the gastrointestinal (GI) tract has been studied in depth. This is not surprising since the majority of the dosage forms are designed for oral administration. Targeting in the GI tract is mainly to achieve improved absorption of a drug and thus bioavailability. As described in Table 10.2, a number of gastric retention devices have been proposed. The role of gastric retention devices is to maintain the oral dosage forms in the stomach for a prolonged period of time. In this sense, the gastric retention devices may be considered as a vehicle for targeting to the stomach and upper small intestine.

B. TARGETING TO SMALL INTESTINE

It is more difficult to target oral dosage forms to a certain region in the small intestine. A number of lectins have been used to target oral dosage forms to small intestine since the surface of intestine is covered with a mucus layer composed of mucins (which have numerous carbohydrates). Techniques for targeting to a selected region of the small intestine still remain to be discovered.

1. Oral Vaccination

Targeting in the GI tract becomes critically important for oral vaccination. Many antigens are taken up the by the Peyer's patches in the intestine to result in mucosal and systemic immunization (Figure 11.4). Thus, the success of oral vaccination depends on the ability to target microparticles to the M-cells in the Peyer's patches or to the vicinity of the Peyer's patches. Antigens are usually loaded in microparticles to protect them from the low pH of the stomach and enzymatic degradation in the intestine. It has been proposed that microparticles with less than 10 μm in diameter are preferentially taken up by the Peyer's patches. Antigens are usually loaded into microparticles of sodium alginate or poly(glycolic–co–lactic acid). The original report on particle uptake by Peyer's patches indicated that the uptake efficiency increased as the particle hydrophobicity increased. Hydrophobic microparticles made of polystyrene showed the improved uptake as compared to hydrophilic microparticles. Based on this observation, hydrophobic liposomes were used for improved oral vaccination. The uptake of polymerized liposomes was about 3%, and this increased dramatically to 10% upon grafting of lectins to the liposome surface. Lectins, cholera toxins, and diphtheria toxoid are known to bind to the surface of Peyer's patches to maximize the absorption.

Not only proteins but also DNA molecules can be used for oral vaccination. Biodegradable polymer particles containing a DNA vaccine were administered orally, and it produced both a blood serum response and mucosal antibodies against the rotavirus in the mice. The mice with the DNA vaccine had lower levels of the rotavirus than controls when

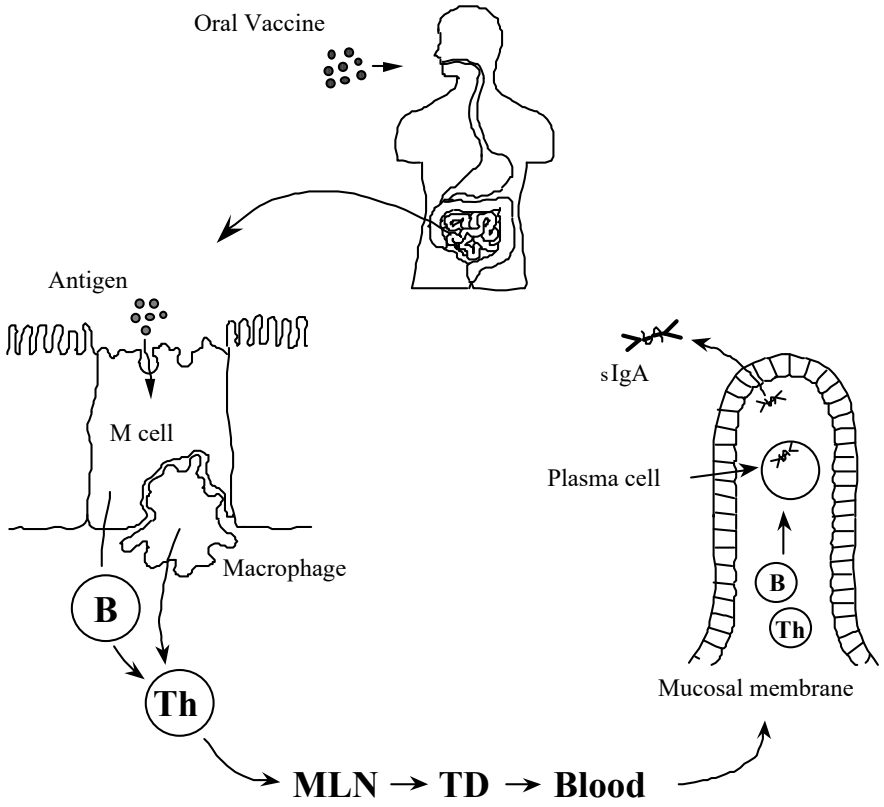

Figure 11.4 Mucosal immunization by oral vaccination. After oral vaccination, an antigen is taken up by M cells in the Peyer's patch of the gut-associated lymphoid tissue. The antigen is then passed to the macrophages and B cells (B). These cells in turn present the antigen to T helper lymphocytes. These cells migrate into the blood via the mesenteric lymph nodes (MLN) and the thoracic duct (TD). These cells subsequently localize in the effector sites, i.e., mucosal membranes of the gastrointestinal tract, upper respiratory tract, genitourinary tract, and glandular tissue. At the effector sites, the migrating B cells develop into plasma cells which produce IgA antibodies. Polymeric IgA is then released as secretory IgA (sIgA) through epithelial cells.

challenged with the rotavirus 12 wk after the immunization (Chen *et al*., 1998).

2. Oral Vaccination using Edible Vaccines

Delivering antigens in fruit, vegetable, and other food is theoretically possible, but packaging an edible vaccine has not been practical. Recently, genetically engineered potatoes were used in human clinical trial to test whether the raw potatoes could induce immune response (Tacket *et al*., 1998). The potatoes were engineered to express LT-B, an enzymatically inactive enterotoxin secreted by *E. coli*. The transgenic potatoes stimulated an immune response in both blood serum and the intestinal mucosa. The uncooked potatoes, however, also caused mild nausea in some subjects, and were not very palatable to kids for whom the vaccines are developed. In another study in the mice, uncooked potatoes containing weakened cholera toxin produced blood serum and mucosal immune re-

sponses. Cholera-related diarrhea in the mice was reduced by about 60% (Arakawa *et al*., 1998). Since cooking potatoes is likely to destroy most of the antigens, fruits that do not require cooking, such as banana, would be the better candidate. The choice of the carrier, however, is based on the ease of transformation and the short generation time of the genetically altered food.

The successful transformation of fruits and vegetables into vaccine factories depends on understanding of the candidate plant's genomics, such as behavior of a promoter in the fruit. The real barrier to edible vaccines, however, is their delivery in the GI tract. The antigens, which are proteins, have to survive the harsh conditions, such as low pH in the stomach and the presence of enzymes throughout the GI tract.

C. Targeting to Colon

Oral dosage forms targeting to the small intestine are yet to be developed. Targeting to colon, however, is rather easily achieved. Since all oral dosage forms, except those with gastric retention platforms, pass through the GI tract in several hours, time-controlled targeting may be achieved. In a time-controlled oral delivery system (*e.g*., Pulsincap®), drug release is delayed until a predetermined time after administration during that the system reaches a target site in the GI tract. Pulsincap® (RP Scherer Corp.) is a brand name for a new oral drug-delivery system that releases drug in the body in a pulsed manner at a predetermined time (or at a predetermined site in the GI tract). Thus, it is a site-targeted system in the GI tract based

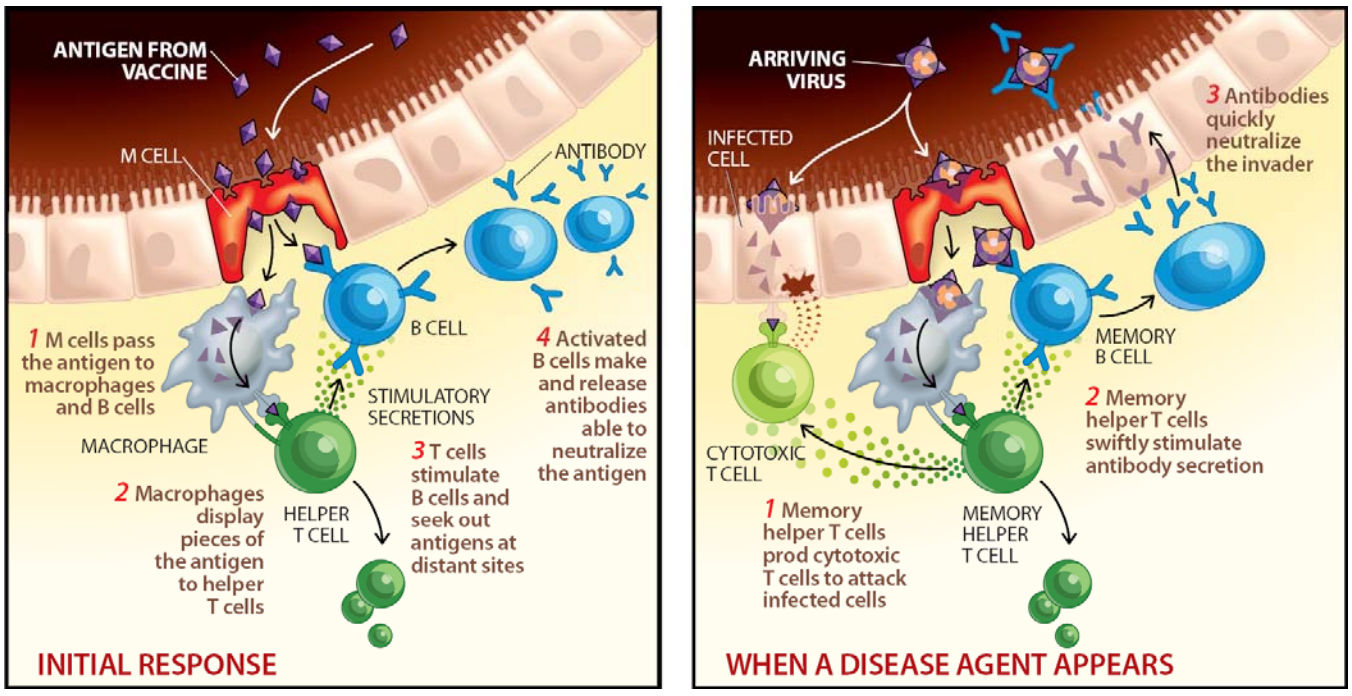

Figure 11.5 (left) An antigen in a food vaccine gets taken up by M cells in the intestines and passed to various immune-system cells, which then launch a defensive attack—as if the antigen were a true infectious agent, not just part of one. (right) That response leaves long-lasting "memory" cells able to promptly neutralize the real infectious agent if it attempts an invasion (Langridge, 2000).

on time-control. It consists of a hollow, insoluble capsule body that is sealed at one end. The other end of the capsule is sealed off with a hydrogel plug that swells on contact with water and is expelled at a certain critical swelling volume. It has applications for both nocturnal and colonic delivery. When captopril was delivered using this device, the drug was delivered to the colon in six out of eight subjects and to the terminal ileum in the remaining two subjects. Diclofenac Pulsincap® is in the development stage for the twice-daily treatment of arthritis. It can be administered orally and releases greater levels of the drug in the early morning hours, when pain and stiffness are worst for arthritis sufferers. Diclofenac is sold by Ciba-Geigy under the brand name Voltaren®.

Other advanced colon delivery systems are based on polymers that undergo degradation by the enzymes present in the colon. Azopolymers are known to be degraded only by bacterial enzymes in the colon. The dosage form can be coated with enteric materials for protection in the stomach and azopolymers for protection in the small intestine until the system reaches the colon. The Targit® technology (by DanBioSyst UK Ltd., Nottingham, UK) utilizes starch capsules coated with enteric polymers and azopolymers. The therapeutic activity of salicylazosulfapyridine is improved by the presence of colonic flora. Splitting of the azo linkage of the drug increases absorption.

IV. GENE DELIVERY

Understanding on the genetic basis for congenital and acquired diseases has been advanced significantly in the past decade, and such an increased comprehension resulted in the ability to modulate gene expression in host cells by introducing genes that encode native or foreign proteins or antisense oligonucleotides. Antisense oligonucleotides are short synthetic DNA segments which are capable of entering cells and hybridizing to mRNA. The hybrid formation inhibits the translation of a gene by causing steric hindrance of the protein translation. As a result, the cells lack a specific protein without affecting the translation of other cellular proteins. Antisense is currently used to investigate the physiological or pathological function of individual genes without genetic manipulation. Gene therapy is a new generation of therapeutic approach which has the most exciting potential in curing various diseases.

Since the death of Jesse Gelsinger, an 18-year-old man who died on September 17, 2000, while participating in one of gene therapy clinical trials by the University of Pennsylvania's Institute for Human Gene Therapy, the gene therapy clinical trials have been under increasing scrutiny by the U.S. Food & Drug Administration (Halim, 2000). Gene therapy is still in its infant stages, and the first clinical trial was made only 10-y ago. The gene therapy research has to go on, but the highest standards of scientific investigation and regulatory/reporting requirements have to be met for clinical investigations.

The success of gene therapy depends on, among many factors, targeting of genes or antisense nucleotides into the target cells. In this particular case, targeting means not only delivery to the target cell but also delivery

into the nucleus and insertion of a delivered gene or an antisense oligonucleotide into the right place. The two most important factors in gene therapy are efficient vectors for entering into the cells and delivery systems that brings the vectors to the target cells.

A. VIRAL VECTORS

Until recently, viral vectors, owing to their relatively high transduction efficiency, have been the predominant method for delivering the payload of therapeutic genes and oligonucleotides into the cells. Commonly used viral vectors are adenovirus, retrovirus, adeno-associated virus (AAV), and herpesvirus. Viral vectors present a few advantages. For example, adenovirus can be grown to high titers *in vitro*, infects a wide variety of resting and growing cells *in vivo*, and accommodates reasonably large foreign DNA inserts (see Figure 11.6). The transgene expression of adenovirus, however, is known to be transient (*i.e.*, expression of reporter genes in various tissues of immunocompetent hosts decreases to undetectable levels in about 3 wk after infection).

Although some viral vectors have demonstrated higher levels of expression and genomic incorporation during clinical trials than nonviral vectors have, they present a number of problems. The main factor that impedes clinical development of vectors is validity and safety aspects. In 1999, gene therapy was used to treat a young man who suffered from orni-

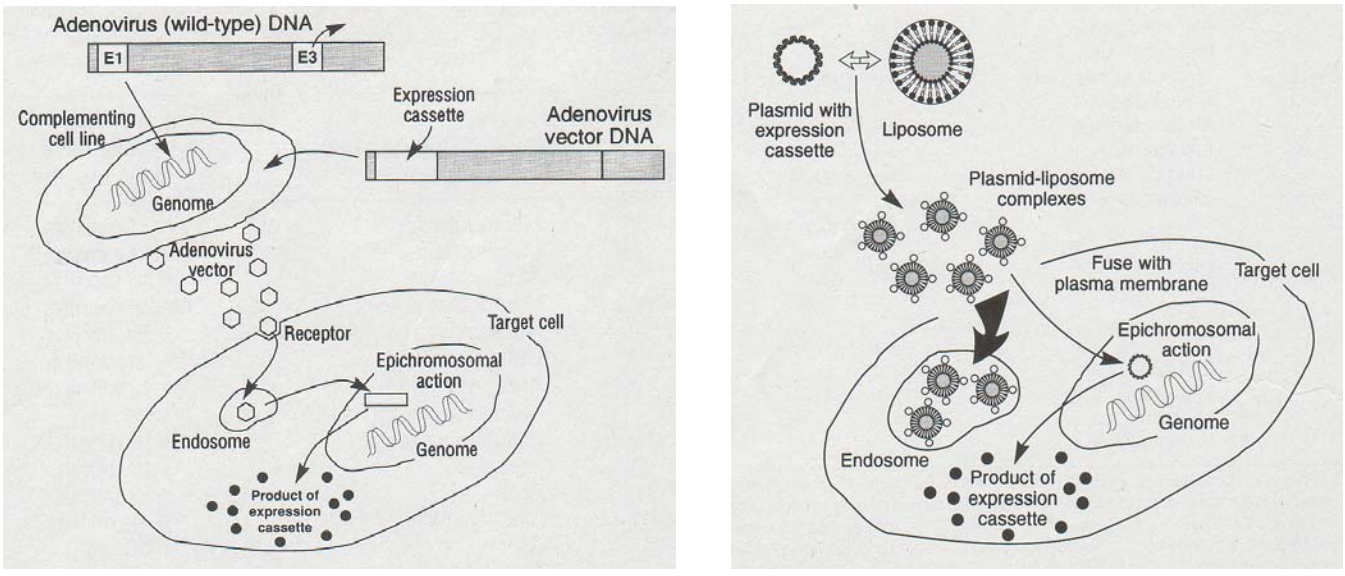

Figure 11.6 Comparison of the viral and non-rival vectors in gene transfection process. (Left) The vector binds to the target cell through an interaction of the adenovirus fiber and penton, each to a specific receptor; moves into a cytoplasmic endosome; and breaks out and delivers its linear, double-stranded DNA genome with the expression cassette into the nucleus, where it functions in an epichromosomal fashion to direct the expression of its product. (Right) The positively charged liposome is complexed to the negatively charged plasmid with its expression cassette. The complexes enter the target cell by fusing with the plasma membrane. The vector does not have an inherent macromolecular structure that conveys information to enable efficient translocation of the plasmid to the nucleus. Consequently, most of the newly introduced genetic material is wasted as it is shunted to cytoplasmic organelles. When used in vivo, it is likely that most, if not all, of the plasmids that reach the nucleus function in an epichromosomal fashion (Crystal, 1995).

thine transcarbamylase deficiency, a genetic disorder that the body is unable to remove ammonia, a byproduct of protein processing. He died during a gene therapy experiment, and it was confirmed that he was killed by the treatment. The most likely reason for the death was that infusion of a weakened cold virus containing corrective genes triggered an extreme immune-system reaction that caused multiple organ failure.

The use of viral vectors to insert the genes or oligonucleotides into the target cells brings more questions, such as possible replication of the virus and possible insertion of additional genetic material of the virus. Viruses have evolved specialized mechanisms for cellular penetration, but some may disrupt human DNA. It is also possible, as exemplified by the incidence mentioned above, that weakened viruses might mutate to regain their pathogenic activity and that the host's immune system may respond to destroy the virus and treated cells. To avoid potential problems associated with using virus, nonviral vectors have been developed.

B. NONVIRAL VECTORS

Nonviral vectors are not as efficient as their viral counterparts, but they avoid triggering an immune response from the body, which may be the biggest obstacle for gene therapy to succeed (Anderson, 1998). This is especially important, since treatment of many conditions by gene therapy will undoubtedly require repetitive treatments. There are growing concerns regarding the safety, efficacy, and immunogenicity of viral vectors, and this prompted the development of nonviral vectors. Nonviral approaches to gene therapy require three key elements:

- Gene that codes for a specific therapeutic protein
- A plasmid-based gene expression system that can modulate both the duration and expression levels of the therapeutic protein
- A gene delivery system that targets the gene to an appropriate tissue/cell.

Of these, development of effective delivery system becomes the most critical factor for the success of gene therapy.

1. Requirements of Nonviral Vectors

The ideal gene carrier should consist of therapeutic DNA, DNA-condensing component, cell targeting moiety, endosomal disrupting moiety, and nuclear translocation moiety. Most of the nonviral vectors do not have all these components in one system.

a. Self-assembly of Artificial Plasmid DNA (pDNA) Vectors

Gene transfer methods using nonviral vectors require self-assembly of artificial plasmid DNA. A critical step in this self-assembly process is the compaction of DNA into particles that are small enough to be injected into patients. The DNA has to be condensed into a small package, and the vectors have to be targeted to specific tissue (via cell surface receptors) without nonspecific uptake.

b. Protection of DNA in Circulation

The ideal gene delivery systems should be able to protect DNA from circulatory nucleases and to exhibit extended circulation lifetimes for accumulation at sites of disease, and to promote intracellular delivery into target cells (effective intracellular trafficking).

c. Escape from Endosomes

Once inside the cell, vectors have to escape the endosome and cross the nuclear membrane for the gene therapy to be effective.

d. Low Cost

Developing actual products is different from having a successful animal model demonstration of gene therapy or even having successful clinical experiment. Pharmaceutical industry has to consider the cost and ease of manufacturing in addition to efficacy.

2. Examples of Nonviral Vectors

a. Cationic Vectors

Since DNA molecules are polyanions, rational choices for gene delivery have been cationic molecules, such as synthetic polycations, cationic lipids, cationic liposomes, cationic proteins, lipophilic cationic peptides, and cationic gelatin microparticles.

The first nonviral vector used was a cationic lipid, named Lipofectin® by Philip L. Felgner, (GeneMedicine: Woodlands, TX) has broad patent rights for the gene therapy use of any cationic lipid complexed with DNA for administration by injection or inhalation. Cationic lipids are widely utilized in nonviral gene therapy, which provides improved efficiency for delivering genes to targeted cells.

Much improvement in transfection activity has been achieved with cationic nonviral vectors. However, their efficiency of transfection is still low compared to viral-based vectors. They are also unable to target specific cells. They may also elicit inflammatory response at moderate doses (see Figure 11.7).

b. Other Vectors

Human artificial microchromosomes, recombinant bifunctional fusion proteins (consisting of a single-chain antibody targeting moiety and a human DNA binding moiety), and inert microparticles (*e.g.*, colloidal gold particles) coated with DNA have also been used.

c. Naked DNA

Nonviral approach to gene therapy can also be used to deliver "naked DNA." This technology arose from the serendipitous discovery that DNA by itself provided expression levels similar to or greater than lipid formulations in some cases. The naked DNA was employed as a negative control in lipid-mediated transfections in the cystic fibrosis gene therapy. Vical Inc. (San Diego, CA) patented the "naked DNA" technology as well as

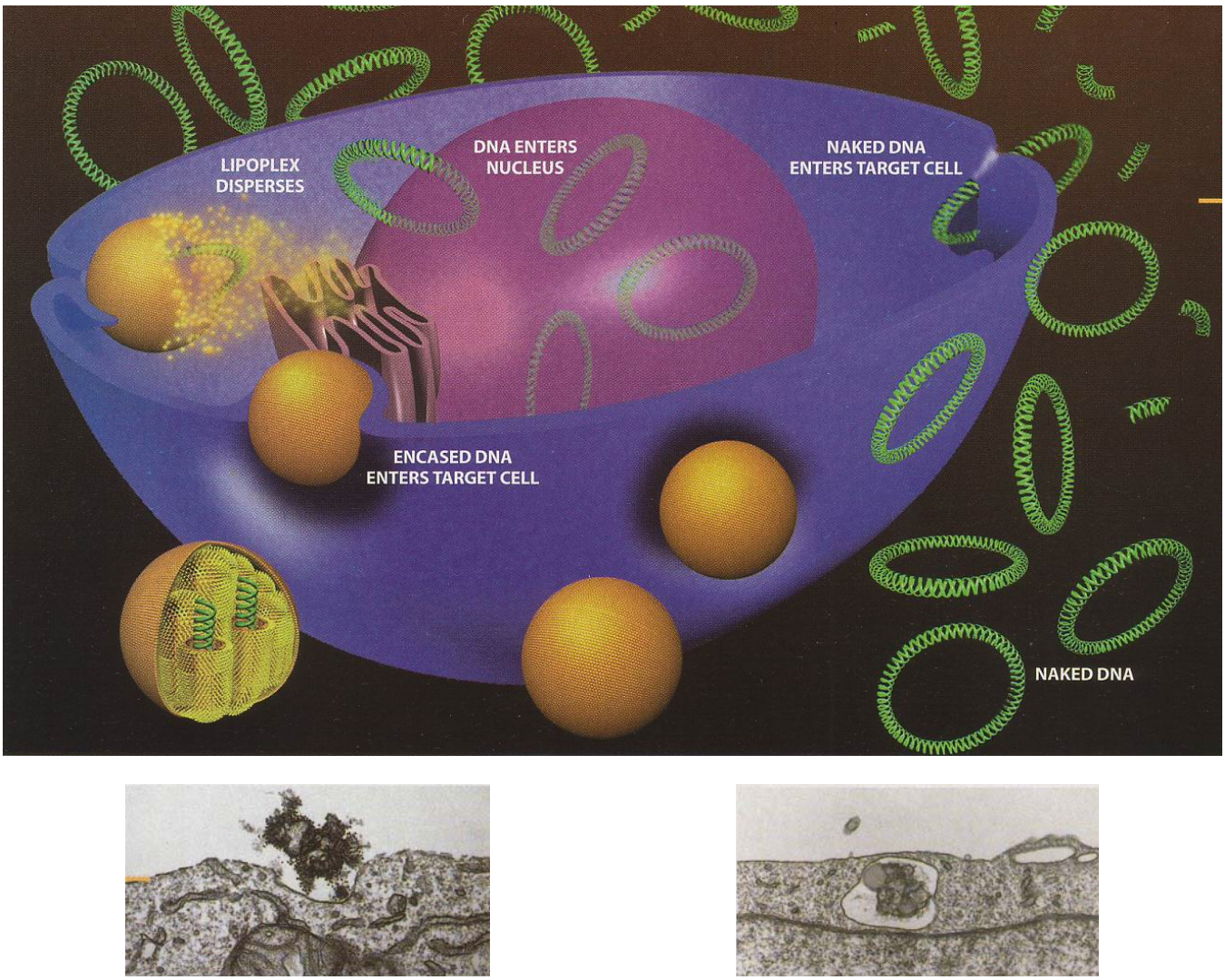

Figure 11.7 (top) Lipoplexes form spontaneously when the genes (*e.g.*, bacteria plasmids) mix with lipids. (bottom) Assimilation of a lipoplex by a cultured cell. The lipid in the lipoplex protects its DNA cargo (Felgner, 1997).

proprietary "cytofectins" (synthetic fatty substances). It should be noted that naked DNA or traditional lipid approaches are nonspecific and/or very low in gene expression.

Aside from delivering therapeutic genes, gene therapy can also be used for the development of DNA vaccines. Introduction of a gene for a pathogenic protein into the body would allow expression of a small amount of foreign protein, as from HIV or malaria, and could stimulate a powerful protective immune response

V. TISSUE ENGINEERING

The loss or failure of an organ or tissue is one of the most frequent, devastating, and costly problems in health care. Every year a large number of people suffer organ and tissue loss from accidents, birth defects, hereditary disorders, and diseases. Current treatment modalities include transplanta-

Table 11.2 Gene Therapy Clinical Trials Worldwide

Vector	Open Trials	Examples of Diseases
Viral		
Retrovirus	157	Many cancers, AIDS, severe combined immunodeficiency, rheumatoid arthritis, graft-versus-host disease, multiple sclerosis, osteodysplasia, hemophilia
Adenovirus	132	Many cancers, peripheral artery disease, cystic fibrosis, Canavan disease
Pox virus	35	Many cancers
Adeno-associated virus	7	Prostate cancer, cystic fibrosis, hemophilia B
Nonviral		
Lipofection[a]	57	Many cancers, cystic fibrosis, coronary artery disease, restenosis
Naked DNA	47	Many cancers, peripheral artery disease, coronary artery disease, peripheral neuropathy, open bone fractures
RNA transfer	5	Many cancers
Gene gun[b]	4	Melanoma, sarcoma

[a] Includes liposomes and various packages of lipid, polymer, and other molecules. [b] DNA coated on small gold particles and shot with a special gun into target tissue (Ferber, 2001).

tion of organs, surgical reconstruction, use of mechanical devices, or supplement of metabolic products. One of the best options for treating ailing tissues and organs is to have organ transplantation. There is, however, serious shortage of the donated organs. Furthermore, a mismatch of tissue types in standard organ transplantation requires lifelong immunosuppression with attendant problems of graft rejection, high drug therapy costs, and the potential for the development of cancer. Using one's own tissues may solve the problem of graft rejection, but the types of tissues available (*e.g.*, small intestine) for reconstruction are often unsuitable. In addition, it requires surgical invasion of another part of the body with functional losses at the donor site. To solve these problems, a new discipline called "tissue engineering" has been emerged that focuses on custom-design and creation of "replacement parts" that can be grafted into humans.

Tissue engineering is a discipline of creating man-made tissues or organs, known as neo-organs (Mooney & Rowley, 1997). Tissue engineering applies the principles of biology and engineering to the development of viable biological substitutes (or artificial template) that replace, maintain, or improve the function of human tissues (Nerem, 1991; Langer & Vacanti, 1993). The engineered tissue becomes integrated within the patient own tissues for cure of the specific disease state. Tissue engineering can potentially be used to make almost all kinds of tissues and organs. The ultimate goal of tissue engineering is to make replacement heart, or even replacement heart parts, such as heart valves. This form of therapy differs from standard drug therapy in that the engineered tissue becomes integrated within the patient, affording a potentially permanent and specific cure of the disease state. Tissue engineering, along with gene therapy, may well dominate medical therapeutics in the future.

A. TEMPLATES/SCAFFOLDS FOR TISSUE ENGINEERING

The main focus of tissue engineering is the restoration and repair of damaged tissues. This can be accomplished by synthesis of artificial constructs or tissues derived from vital cells or cell matrix, or better "directing" the host repair processes with "informational" scaffolds. The key components in tissue engineering are scaffolds, specific signals, and cells (Mooney & Rowley, 1997). Scaffolds/matrices, made from various (preferably biodegradable) biomaterials, provide a three-dimensional structure (or physical support) to shape or guide the development of a desired tissue structure from cells that may be seeded within these porous structures or migrate from the surrounding host tissue.

The scaffolds/matrices for cell transplantation can be fabricated in a variety of configurations from naturally occurring extracellular matrix (ECM) molecules (*e.g.*, collagen), ceramics, and synthetic polymers (poly α-hydroxyesters and polyanhydrides). Currently, both nonresorbable and resorbable bioinert matrices that mimic the shape of living tissue are implanted. To aid the adherence and growth of desired type of cells, cell-adhesive proteins are immobilized onto the matrix surface. To engage in true tissue engineering that allows us to grow a number of tissue types at will, matrices need to contain specific morphogenic proteins capable of directing new tissue growth. To accomplish this, we need to know exact conditions for cell growth. For example, it is necessary to know the specific concentrations (and concentration gradients) of the molecules or substances that are necessary for the desired effect in shaping organ regeneration. It is also necessary to know how long the cells should be exposed to such substances, and when one factor needs to be replaced by another. A critical challenge in engineering neo-organs is supplying nutrients to every cell growing inside of the scaffolds/matrices. Supplying nutrients and oxygen, at the same time removing cell metabolites, may require new blood vessels growing into neo-organs. Adding angiogenesis-stimulating molecules inside the scaffolds may be necessary, in addition to adding cell growth factors. In this endeavor, the controlled release technology would be vital.

B. APPROACHES IN TISSUE ENGINEERING

There are at least two approaches in the preparation of artificial templates: acellular templates; and cell-matrix tissue equivalents. Acellular materials can be remodeled by like tissues adjacent to an implant in the body. The objective is to provide both matrix substance and biomolecules that induce and signal cell migration, cell propagation, and cell differentiation (Bell, 1991. The design of the matrix can be manipulated by modifying the content of signaling and regulating molecules (Bell, 1991). For practical applications, this acellular template approach may be better than the cell-matrix tissue approach described below, since the former does not require any extra steps such as isolation of cells and seeding the isolated cells.

Cell-matrix tissue equivalents, also called cell-polymer construct (Vacanti *et al*., 1991), can be built to serve as substrata for specific cells, such

as human microvascular endothelial cells and smooth muscle cells. These cells can constitute simple or stratified tissues and depend for their differentiation on diffusible support molecules from cells in the matrix and from the media provided. The matrix itself is usually made of degradable polymers such as poly(lactic–co–glycolic acid) or collagen, and is usually enriched by cell adhesive molecules. In this approach, it is required to have a source of cultivatable cells and methods for removing cells that are known to provoke an immune response (Bell, 1991. Either of the above two approaches has been used to prepare a variety of tissue equivalents such as heart valve leaflets, liver support systems, dermal tissues, cartilages, and nerve guide.

C. Preparation of Porous Scaffolds Used in Tissue Engineering

Scaffolds are usually made of biodegradable polymers since the templates are expected to be replaced by the natural tissues eventually. Both natural and synthetic polymers have been used in the preparation of scaffolds. Widely used natural polymers are collagen, collagen-glycosaminoglycan graft copolymers, fibrin gel, gelatin, agarose, and alginic acid. A number of synthetic polymers have been used and are currently being developed. PLA, PLGA, PGA, poly(β-hydroxybutyrate-β-hydroxyvalerate) are most commonly used, while new degradable biomaterials, such as pseudo-poly (amino acids) and polyphosphazene are under development. Soluble scaffolds can also be prepared from poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG) and their blends or copolymers. The advantage of such systems is that they can be tailor made so that they dissolve very fast, slowly or very slowly and be used as otherwise inert scaffolds. Also because of the method of preparation they are easy to cast in various shapes.

A biodegradable and biocompatible polymeric support for tissue ingrowth should have high porosity (with pore diameter in the 10–100 μm range), interconnected pore network, and good mechanical strength and flexibility (Schugens *et al*., 1996). The porous matrices can be prepared by various methods. Methods used in the tissue engineering area are salt-leaching (Mikos *et al*., 1994), phase separation (Lo *et al*., 1994), emulsion freeze-drying (Whang *et al*., 1995), spray drying (Lo *et al*., 1996), and microparticle aggregation (Schugens *et al*., 1995), to name several. The preparation of the matrices with homogeneous with interconnected pores by salt-leaching method requires 70–90 (w/w)% salt, and the size of the salt particles play an important role in determining the size of pores. The phase separation methods utilize polymer precipitation in a poor solvent such as water. Since water can also be used as a porosigen, the pore size can be easily controlled from less than one micrometer to larger than several hundred micrometers. Other methods also provide conveniences in controlling the porosity. Each method has its own advantages and limitations, and the choice of the method depends on the type and nature of the final matrices to be made.

Porous matrices need to be constructed in precise anatomical shapes for replacing tissues. Three-dimensional shapes of biodegradable, porous matrices have been made by lamination of thin layers of porous membranes

(Mikos *et al*., 1993; Bellamkonda *et al*., 1995), spray casting on a Teflon mandril for hollow organs (Grower, Russell, & Cutright, 1989), or connecting biodegradable meshes to a certain shape (Mooney *et al*., 1996).

D. ISSUES IN TISSUE ENGINEERING

There are several key components necessary for successful tissue engineering as listed in Table 11.3. The exact requirements for successful tissue engineering depend on the nature of application. For example, in tissue engineering of blood-contacting materials, Greisler and co-workers (Greisler *et al*., 1996) have suggested that it is possible to modulate the relative proliferative activity of endothelial cells (ECs) versus smooth muscle cells (SMCs) by altering the ratio between FGF-1 and heparin. These signals necessary for the regulation of EC and SMC proliferation may be different from the signals for the proliferation of other types of cells. In addition to the tissue growth potential, nonautologous conduit vascular grafts should ideally be nonthrombogenic, stiff but flexible enough for easy suturing.

Since various regulatory signals in ECMs may work synergistically, adding one or a few known regulatory signals is not able to reproduce the unique abilities of ECMs. For this reason, it is critically important to find out the type and concentration of the regulatory signal molecules involved in the adhesion, proliferation, and differentiation of ECs and SMCs. This information will be obtained in other components of this collaborative proposal. It is equally important to identify chemical, physical, and structural characteristics that provide the optimal cell behavior in contact with the scaffold. Particular emphasis must be placed on the nature and the structure of the porous architecture of the scaffolds, as it controls the tissue ingrowth. The three-dimensional distribution of the polymer scaffold must be optimized for best cellular and tissue ingrowth. All these important aspects of tissue engineering will be dealt with in this project.

Table 11.3 Factors Important in Tissue Engineering

Regulatory signals (*e.g*., growth factors) inducing growth of specific cells.
Optimal combination of regulatory signals to initiate and promote tissue growth
Maintenance of cell functions
Synthesis of an appropriate extracellular matrix by cells
Cell alignment (*e.g*., alignment of endothelial cells exposed to fluid shear stress)
A scaffold for new tissue to form.
Controlled porous architecture for cellular or tissue ingrowth
Three-dimensional shape
Distribution and release (either spatial or temporal) of regulatory signals
Strength, durability, and longevity appropriate for location and junction
Nonthrombogenic property for blood contacting surfaces
Host cells capable of differentiation into tissue.
Vascularization

E. Examples of Tissue Engineering Products

1. Artificial Skin

Integra LifeSciences Corp. (Plainsboro, NJ) is marketing an *in vivo*, non-biologic regeneration product, Integra® Artificial Skin, approved by the FDA in 1996 for the treatment of life-threatening burns. The artificial skin consists of an outer layer of silicone rubber and an inner layer of bovine collagen and a shark-cartilage derivative. The inner layer induces the patient's dermis to regenerate and is later reabsorbed by the body. The outer layer seals in moisture for 2–3 wk, after which a surgeon peels it off and covers the wound with a thin graft of the patient's own epidermis. Integra's product has been shown to cause significantly less scarring than the traditionally used full-skin graft.

2. Bone-Grafting Material

In early 1970s, electron microscopists first noticed similarities between human spongy bone and coral. In 1993, Interpore International (Irvine, CA) introduced a bone-graft material derived from coral, dubbed Pro Osteon™, which was the first such material approved by the FDA. Pro Osteon™ is molded into the void created by a serious bone fracture or defect to form an "osteoconductive" scaffold into which the body's own bone grows.

Charles Vacanti, Director of the Center for Tissue Engineering at the University of Massachusetts Medical Center in Worcester, MA, grew patient's bone cells and injected them into a piece of coral fashioned into the shape of the missing digit. The coral degraded as bone replaced it. The patch was then implanted back on the thumb to grow the missing bone.

3. Blood Vessels

Tissue engineering of blood vessels presents a special challenge, since they have to be strong yet flexible enough to expand and contract with each heartbeat. The tissue-engineered vessels have to have the endothelium to prevent thrombosis. Muscle cells can be grown around a polymer tube, and the endothelial cells can be seeded or closely packed lining cells can be added inside the tube. The ideal tissue-engineered vessel may be the one that has a well-defined, three-layered histological architecture, complete with an intima, a media and an adventitia.

4. Cornea

Lines of cultured human cornea cells were used to fashion the first working equivalent of a human cornea (Griffith *et al*., 1999). The artificial cornea was constructed by first growing a monolayer of endothelial cells on a plastic culture dish. The endothelial monolayer was covered with a mixture of keratocytes (which populate the stromal layer). The scaffold used for growing the stromal layer was made of a collagen-chondroitin sulfate crosslinked with 0.02–0.04% glutaraldehyde. Unbound glutaraldehyde was removed by treating with glycine. Finally the epithelial cells were layered on top. To make three lines of cells immortal (*i.e*., grow in-

definitely), they were infected with viral genes. The cells of the engineered cornea may become cancerous later on and/or cause immune response after implantation. In addition, the cornea may not remain transparent. This engineered cornea, however, is the first step toward making off-the-shelf corneas for human transplants.

This particular example shows how the gene therapy and tissue engineering can be combined to produce new tissues/organs that can be transplanted.

5. Stem-Cell Transplant

Stem cells are the most immature human cells ever discovered. Stem cells are self-renewing elements that can generate many different cell types in the body (Lumelsky *et al*., 2001). Stem cells are found in fetal tissues as well as in adult, but the embryonic stem cells (those derived from an early stage of the mammalian embryo) have the widest development potential. The embryonic stem cells can be induced to differentiate into cell types found in the blood, heart, muscle, brain, and pancreas (see Figure 11.8). For this reason, the stem cell research holds the key to treatments of Alzheimer's disease, heart disease, neurological disorders, Parkinson's syndrome, and diabetes, to name a few. In a stem-cell transplant, isolated stem cells can be cultured and seeded into a polymer molded to fit an injured area, for example, damaged knee cartilage. The polymer scaffold then can be inserted into the patient when the cells were still undifferentiated. The cells, responding to locally acting endogenous factors, would differentiate appropriately and form the correct tissue structures. Autografts are fully differentiated tissues transplanted from one area of the body to another. So far, bone and skin have been used. If scientists could only figure out how to provide stem cells the right stimulation, each stem cell could become a different organ or tissue.

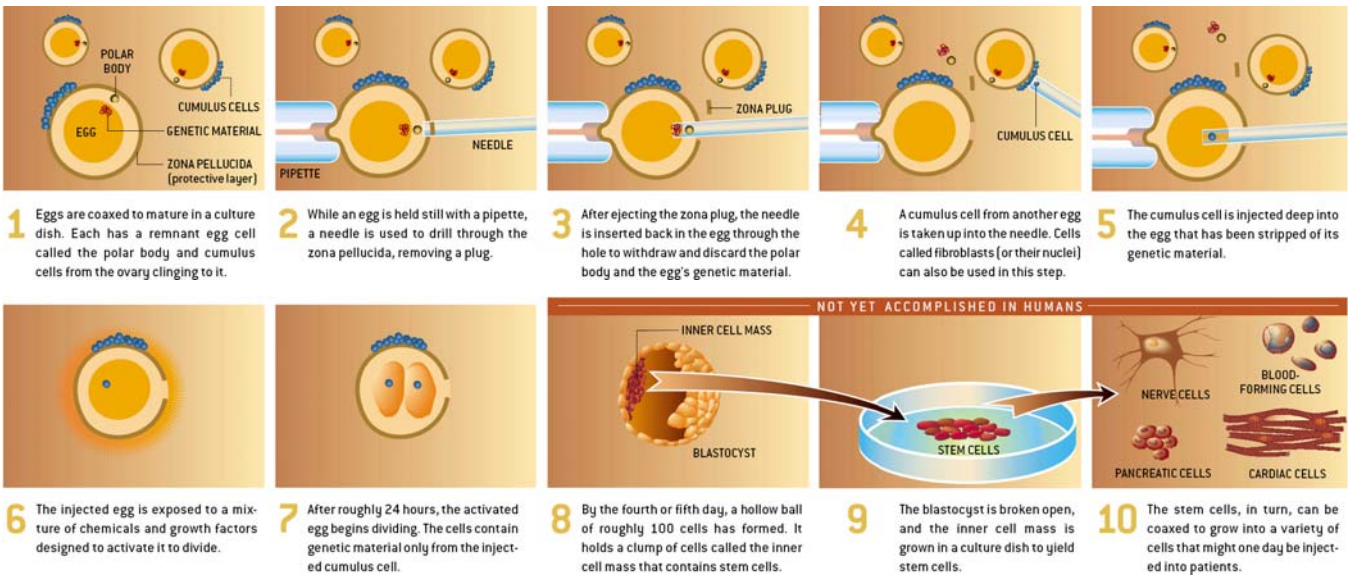

Figure 11.8 How Therapeutic Cloning is done (Cibelli *et al*., 2002).

Gene Delivery—without Viruses

(Henry, 2001)

Nonviral methods represent only a fraction of the gene delivery field, but they are catching up with viral vectors.

For more than 20 years, researchers have been working to alleviate disease through gene therapy. In this type of treatment, a gene is delivered to cells, allowing them to produce their own therapeutic proteins.

For example, many researchers have focused on inserting genes for Factor VIII to treat hemophilia A and Factor IX for hemophilia B. Hemophilia is a popular model system because it is a single-gene defect, and replacing about 5% of the amount of protein present in normal individuals is enough to effect a therapeutic cure.

At the beginnings of gene therapy, genes were usually transferred by using viruses that could infect cells, deposit their DNA payloads, and take over the cells' machinery to produce the desirable proteins. Increasingly, however, researchers are ditching the viruses and using plasmids--small rings of DNA produced in bacteria--to get the genes into the target cells.

In the past, such nonviral methods of gene delivery--including "naked" DNA and DNA condensed with agents such as cationic lipids or polymers--were considered less efficient than viral vectors. (Naked DNA is simply plasmid DNA not complexed with anything else.) But industry and academic researchers working in the nonviral gene delivery field say that's not true anymore. Despite improvements, however, nonviral methods remain a small, though increasing, fraction of the research presented at gatherings such as the annual meetings of the American Society of Gene Therapy.

A number of the people working in nonviral gene therapy used to work with viral vectors. They had different reasons for turning to nonviral methods, but they do have one thing in common: They saw problems with viral gene therapy.

Jon A. Wolff, director and professor of pediatrics and genetics at the University of Wisconsin, Madison, Medical School, used to work with retroviruses, which carry their genetic material in RNA instead of DNA, as a way to deliver genes. But then he realized that viruses had some limitations. "I thought nonviral might be a simpler and easier way of [effecting delivery] and avoid the problems with viral" therapy, he says. The main problem is the immunogenicity of the viruses. "Viruses have evolved over a billion years to efficiently deliver DNA into our cells, but our immune system has also evolved over that time," he says.

Wolff also saw nonviral methods as an opportunity to use his undergraduate training in chemistry. "It seemed to be more of a molecular chemistry sort of challenge than working with [viral] vectors" was, he says.

Richard F. Selden, president and chief executive officer of Transkaryotic Therapies in Cambridge, Mass., has always worked with nonviral methods. He saw that there could be two basic approaches to developing gene therapy vectors: either taking what was then the state of the art in molecular biology and using that to jerry-build a system that would work for gene therapy or determining the ideal properties of a gene therapy system and then starting from scratch.

Selden took the "ideal properties" approach because he believed that the state of the art in molecular biology was moving so rapidly in the late 1970s that "it didn't make sense to say: 'Let's just stop where we are in 1978 and try to turn that into gene therapy.' "

In addition, Selden worried about the safety of retroviruses, at that time the main way of getting genes into cells. Perhaps the most notorious retrovirus is HIV, the virus that causes AIDS.

"The reason people thought retroviruses were okay was that they said that retroviruses were unassociated with any human disease," he says.

"Even though there wasn't yet an association, there were clear data that should have made people worried. It was shown that a retrovirus, the mouse mammary tumor virus, was the causative agent of breast cancer in mice. Even though I never could have imagined how dangerous retroviruses really are, I don't think it took any great leap of faith to think that retroviruses have safety problems."

Selden has similar concerns today when he hears people say that the most popular viral vector for gene therapy--the adeno-associated virus--is unassociated with human disease. He thinks that viruses are inherently unsafe and should be avoided. "I thought that a nonviral method would allow a safer system and would also ultimately allow more control and would give better efficacy," he says.

"Viral vectors are always going to be handicapped by the regulatory, safety, and production issues," says Philip L. Felgner, chief scientific officer at Gene Therapy Systems in San Diego. With "nonviral methods, if they remain relatively simple carrier systems, it will be much easier to achieve practical pharmaceutical products. It's going to be a yin-yang or tug-of-war between high expression and practical issues."

In developing nonviral vectors, the goal is to design a system that simultaneously achieves high efficiency, prolonged gene expression, and low toxicity, says Leaf Huang, professor of pharmaceutical sciences at the University of Pittsburgh. A number of vectors fulfill one or two of those criteria, but it is difficult to meet all three, he says. The most consistent problem is toxicity, he notes.

Nonviral vectors can be divided into two broad categories--physical and chemical--according to Huang. Physical methods involve taking plasmids and forcing them into cells through such means as electroporation, sonoporation, or particle bombardment. Chemical methods use lipids, polymers, or proteins that will complex with DNA, condensing it into particles and directing it to the cells.

Nonviral systems for gene delivery have several potential advantages over viral vectors. Viruses cause an immune response that can make repeat administrations ineffective. Nonviral vectors also can usually carry more DNA than viruses, allowing the delivery of larger genes.

In addition, nonviral vectors are easier and less expensive to manufacture. The plasmids that are used in nonviral systems can be produced in bacteria such as Escherichia coli. The same production facilities can be used to manufacture a variety of plasmids incorporating different genes. And, as Robert C. Moen, president and CEO of Copernicus Therapeutics, Cleveland, points out, all the other components of nonviral systems are chemical, "so it's like a real pharmaceutical."

Synthetic gene delivery systems can be manufactured as pharmaceutical products, agrees Alain Rolland, senior vice president of preclinical R&D at Valentis in The Woodlands, Texas. "We have been able to manufacture gene-based medicines as single-vial, lyophilized, stable formulations," he says. Some of the products that Valentis is using in its clinical trials have been stable for more than two years at 4 °C and some, even at room temperature. In contrast, many viral vectors must be frozen to be stored for extended periods.

Synthetic delivery systems are also much easier to characterize than viral vectors. "Nonviral systems or gene-based medicines can be characterized as any pharmaceutical product" can, Rolland says. "Depending on the specific formulation, we can characterize the delivery components, the complex size, the DNA concentration, the synthetic gene delivery concentration, and so on. We can use a number of assays for specifications of our products."

Mirus Corp., Madison, Wis., has been working on intravascular methods of delivering naked DNA to muscle in collaboration with Wolff, one of the company's founders. "We inject directly into the vascular system and then increase the permeability of the blood vessel so DNA can escape the blood vessel and target the myofibers of the muscle cells directly," says James E. Hagstrom, vice president for scientific operations at Mirus. The method relies on high volumes and rapid injections to increase the pressure in the blood vessels and allow DNA to "leak" out. In trials with primates, the company used a blood pressure cuff to increase the pressure.

Other researchers have suggested that such a method might not be "clinically relevant." That was also a concern for Wolff, but he thinks Mirus's studies are suggesting otherwise. "We did it in monkeys, and it worked really well and wasn't very toxic," he says. They have also used high-pressure techniques to deliver DNA to the liver in dogs and the heart in pigs. The method shows some toxicity to those organs. "By controlling how high the pressure is and the condition, one can balance the toxicity versus the benefit," Wolff says. "The actual data are looking more promising than I initially thought."

Vical, based in San Diego, is another company that is working with naked DNA. Plasmids are mixed with carriers and injected into the muscle. These carriers don't condense DNA, but they allow the plasmids to get past the cell membrane and into the nucleus, according to Vijay B. Samant, Vical's CEO. In addition, the carriers help protect DNA from nucleases, which are enzymes that chew up nucleic acids. For vaccine applications, these so-called adjuvants help turn on the innate immune response, says David C. Kaslow, chief scientific officer at Vical.

Vical is using naked DNA to develop cancer vaccines. The plasmids express an antigen that is suppressed in the cancer cell. The antigen makes the tumor cells more visible to the immune system, which then attacks them. One product, called Allovectin-7, is in Phase III clinical trials for treatment of metastatic melanoma, a particularly deadly form of skin cancer. "We stand a chance of being the first gene therapy product approved in the U.S., assuming our results come out where they should be," Samant says.

Genteric, Alameda, Calif., is working on a way to deliver naked DNA orally. Although the company calls the concept the "gene pill," President and CEO Martin D. Cleary points out that it doesn't yet "have a pill in the sense of a small white, brown, or pink object." The company is working to develop an oral administration vehicle for DNA. Right now, DNA is delivered to the target cells--the intestinal villi--by a catheter that delivers naked DNA directly to the intestine or by a process called oral gavage, which is the introduction of a fluid to the stomach through a tube.

The intestinal cells that are transfected have a short life: one to two days on average. Therefore, the gene expression is very short term. Cleary calls that the "beauty" of the gene pill. "You have the condition where, if you want a therapeutic effect, you administer a pill. When you want the therapeutic effect to stop, you stop administering the pill, and the protein manufacturing stops almost immediately," Cleary says. Insulin is one of the proteins whose gene they hope to deliver using the gene pill. Other delivery platforms that Genteric is working on include delivery to the salivary glands and the liver.

In another major area of nonviral gene delivery, chemicals such as cationic lipids or polymers are used to condense DNA into particles. These particles tend to range in size from 100 to 300 nm.

One of the challenges for DNA particles is releasing DNA in the cell. Mirus is working on particles as well as naked DNA. "We have the ability to get these particles into [liver cells] efficiently," Hagstrom says. "What we don't have is the ability to get high levels of gene expression from those particles."

The problem, Hagstrom continues, is that you need stable particles that are able to fall apart and release DNA once they're in the cells, so that DNA can get to the nucleus. "We think we've solved a lot of the assembly issues, but now we're working on the disassembly of the particles once they reach their target cell."

Although the mechanism of releasing DNA from the particles is not known, Cyrus R. Safinya, a professor of materials, physics, and biomolecular science and engineering at the University of California, Santa Barbara, proposes that the lipids are peeled away a layer at a time, like an onion, exposing DNA that can leave the complex (Radler *et al*., 1997; Koltover, Wagner, & Safinya, 2001).

Moen claims that Copernicus is able to condense plasmid DNA to "its theoretical minimum size." Most of their work is being done with polylysine derivatives. They can compact an "average size" plasmid to about 20 or 25 nm. Moen believes that this small size allows the particles to get into the nucleus of nondividing cells, with efficiencies three to four orders of magnitude higher than those seen with noncompacted DNA, through

the nuclear pores, which are 25 to 50 nm wide. It is usually thought that DNA enters the nucleus during cell division, because the nuclear membrane breaks down during mitosis. Poly(ethylene glycol) added to the particles helps keep them stable at high concentrations.

"We use the polylysine because, when it's complexed with DNA, it's not toxic. When you use a lipid-based system, often the lipids themselves are associated with some toxicity," Moen says. "Polylysine by itself in large quantities can have toxicity. But when polylysine, which is very positively charged, is complexed with DNA, which is very negatively charged, they combine, and all the toxicities seem to go away for both agents."

Huang's lab has worked to solve the problem of toxicity in using cationic lipids. Rather than premixing DNA and lipid, he injects them sequentially, lipid first. A few minutes later he injects DNA. "We found that the transfection activity in the lung is just as high, if not more, than with the complex," Huang says. "More importantly, the inflammatory toxicity is greatly reduced." His suspicion is that the reduced inflammation results from less DNA being delivered to macrophages, which produce cytokines that cause inflammation. He doesn't believe that such an approach would work for local delivery to tumors.

Targeted Genetics is working on both viral and nonviral methods of gene delivery, with the nonviral methods being focused on shorter term expression. "We like to use [nonviral vectors] in the case of cancer, where you actually want the cell that receives the gene to die," says Ralph W. Paul, director of technology discovery. The company has two different nonviral systems. One is a lipid-DNA complex that is currently in clinical trials for head and neck cancer as well as ovarian cancer. Targeted Genetics' other nonviral system uses cations to condense DNA, which is then placed in a liposome.

Insert Therapeutics is also working with condensing agents. However, the company is working with cyclodextrin-containing polymers rather than lipids or the more traditional polymers. The company's work is based on research done in the laboratory of Mark E. Davis, a professor of chemical engineering at California Institute of Technology.

"At Caltech, we have been working on the structure-property relationships between the molecular structure of polymers that bind and condense DNA and their cellular gene delivery properties," Davis says. "Our work is showing that very subtle structural variations in the polymer can have large effects in the gene delivery properties. The mechanistic origins of these relationships are under investigation as well."

Davis says one of the important things about using cyclodextrin-containing polymers as a gene delivery vector is the intellectual property (IP) considerations. "These delivery products have their own intellectual property and do not require additional IP for application," Davis says. "This will be an important feature in the future, as the IP for fully formulated products that are based on either lipids or polycations for nonviral gene delivery is becoming very messy."

Gene Therapy Systems is developing "peptide nucleic acid-dependent gene chemistry" to improve gene expression. PNA is a DNA analog that has a peptide backbone rather than a sugar and phosphate backbone. PNA can bind to its target sequence on DNA and actually displace the nontarget strand, Felgner says.

"We've made a molecule called a peptide nucleic acid clamp," Felgner says. "It's called a clamp because it has two places that it binds to DNA, two ways that it binds to the same sequence." First, PNA forms a Watson-Crick duplex with DNA. Then, another strand binds to the duplex, so two PNA strands are bound to the same target. The researchers attach various targeting ligands to PNA that possibly help DNA cross the nuclear membrane.

For delivery to skeletal muscle, Valentis is using polymers that interact with DNA without condensing it. Skeletal muscle is dense tissue, without a lot of space for particles to diffuse through, Rolland notes. "We found that, with this noncondensing system, we can retain the flexibility of DNA. The DNA is better able to diffuse through the muscle and get access to a larger number of cells, which translates to higher levels of expression," he says.

These PINC (protective, interactive, noncondensing) polymers have been designed to protect DNA from nucleases. Unlike condensing polymers, PINC polymers don't interact with the phosphate groups in the DNA backbone. Instead, they interact with DNA through hydrogen bonding or hydrophobic interactions. They fit in the major groove and coat the DNA, modifying its surface properties.

"We've been looking at this system mainly for solid tissues, such as skeletal muscle, cardiac muscle, and solid tumors. In solid tissues, you need to keep the DNA in a flexible way so that it can diffuse better through the extracellular matrix and get access to a larger number of cells," Rolland says. "If you condense the DNA in some of these tissues, you don't see very good diffusion and mainly see expression at the site of injection or along the needle track."

The first-generation PINC polymers were polyvinyl derivatives. They are being used in two products that are currently in clinical trials, interleukin-12 and interferon-a for head and neck cancer and melanoma. Other polymers include a poly(oxyethylene)-poly(oxypropylene) block copolymer called poloxamer, which has entered clinical trials with a gene-based medicine for cardiovascular disease.

Safinya is trying to figure out just how DNA-lipid complexes work. "If you look at nonviral gene delivery, it involves using synthetic particles that have been designed based on chemical and physical concepts and then looking at how they interact with a cell," he says. "A lot of those events are physically and chemically dependent on the nature of the assembly."

Safinya's goal is to identify chemical and physical parameters and determine how they correlate with transfection. Such parameters could include the lipid structure, its charge distribution, or the mechanical properties of the membrane. He notes that scientists have virtually total control over the properties of the synthetic complex. "You can begin to design specific molecules that will counteract host mechanisms that are set to destroy anything that comes in," he says.

One of the goals should be to design particles that can target particular cells, much as viruses do, Safinya says. "Viruses have coevolved with cells, so that they will come in and arrest certain natural processes. Then they'll take over the machinery and make thousands of copies of themselves. That's sort of what we're trying to do."

One of the ways to target DNA is to use ligands on the particle surface. Many people are developing such targeting moieties. For example, Copernicus is targeting the polymeric immunoglobulin receptor, which is found in cells lining the airway. Selective Genetics is using fibroblast growth factor as a ligand.

Some companies are working on what is called cell-based or ex vivo gene therapy. For example, Transkaryotic Therapies places therapeutic genes, using an electric field to open pores in the cellular membrane, in cells harvested from a patient. The cells are then reimplanted.

Transkaryotic Therapies primarily uses fibroblasts, which are connective tissue cells. These cells are particularly good at dividing in culture but don't grow much once they're back in the body, Selden says. "Another big advantage of fibroblasts is that when you put them in the body, they stay where you put them," he says. "They lay down a nice collagen matrix." To treat hemophilia, the fibroblasts are implanted in abdominal fat, he says, because the Factor VIII protein is large and is not taken up efficiently when injected subcutaneously.

Transkaryotic Therapies published the results from its Phase I study for hemophilia A this past summer (Roth *et al*., 2001). "We have shown that four of the first six patients with hemophilia A had clinical benefit, with two of those patients not bleeding spontaneously for almost a year," Selden says.

Immune Response Corp. is also working on cell-based gene therapy. They are engineering adult stem cells to produce Factor VIII. They haven't yet figured out how to get the cells to populate the liver, but they're working on it, according to C. Richard Ill, senior director for molecular biology.

Immune Response's shift to stem cells comes after eight years of working on methods to deliver DNA as particles using condensing agents. Although they had little luck with condensing agents,

their concurrent efforts to increase the potency of the Factor VIII plasmid led them to create a novel gene for the protein.

Much of gene therapy is done with complementary DNA (cDNA), which is reverse transcribed from messenger RNA, because the actual genomic sequences are too big. For example, the Factor VIII gene has about 185,000 base pairs. The cDNA has only 9,000 base pairs, because all the noncoding regions, or "introns," have been removed.

The problem with using cDNA is that cells have complexes called spliceosomes that take RNA being produced from DNA and splice out the introns at what are called splice donor and splice acceptor sites. Even though the cDNA is pure coding sequence, the spliceosome still looks for recognition sequences on the RNA and splices part of the RNA.

"We've gone through using computer algorithms and searched for these splice donor and splice acceptor and branch sequences," Ill says. "We essentially said we don't want those sequences in our coding sequence." The DNA sequence is changed so that a different set of nucleotides is used to code for the same amino acid. RNA produced from these altered sequences is more stable, with less likelihood of producing aberrant proteins, he says. This technology could have applications anywhere that cDNA is used, not just gene therapy.

Another technology that may find application in cell-based gene therapy is the human artificial chromosome being developed by the Canadian company Chromos Molecular Systems, Burnaby, British Columbia. These chromosomes have all the features of natural chromosomes, including a centromere, telomeres, and structural, noncoding DNA. However, they contain only the genetic information that Chromos scientists engineer into them. The artificial chromosomes reside in the nucleus beside the natural chromosomes without integrating into the natural chromosomes. When the cell divides, the artificial chromosomes replicate just like natural chromosomes.

Chromos grows the artificial chromosomes from naturally occurring acrocentric chromosomes, which have their centromeres very close to one end, producing short and long arms. "We've been able to identify a sequence that we refer to as the megareplicator," says Alistair Duncan, president and CEO. "This means that when we target into it, it triggers a big amplification. The short arm begins to grow. A second centromere ultimately appears. Now you have a structure that has a long arm of the chromosome, then a centromere, then it has the new chromosome. It does not have any genetic information in it other than what we've targeted in there as part of the triggering process." This dicentric chromosome is unstable, and when the cell divides, it breaks, leaving the original chromosome and the new artificial one.

The artificial chromosomes are large structures: between 40 million and 60 million base pairs long. Chromos has demonstrated information-carrying capacities of between 1 million and 1.5 million base pairs, Duncan says. "Our system allows you to start contemplating working with gene arrays, multiple copies of a gene, or geno-mic sequences. It allows you to start contemplating putting on genes and coupling them to appropriate regulators. It allows you to really start thinking about being able to focus in on novel and complex proteins that might not otherwise be able to be produced in existing systems," he says.

Selective Genetics is carrying out local gene delivery using a technology called gene-activated matrix, which is composed of a biocompatible matrix, according to Barbara A. Sosnowski, vice president for technology development. It can be made of materials such as collagen, a hydrogel, carboxymethylcellulose--any biocompatible material. The matrix is mixed with a gene therapy vector, viral or nonviral.

The mixture can be made to assume any desired shape for use at a wound site. If it is lyophilized, it can form an implantable sponge. It can also be used as a gel that is injected via syringe. As the body tries to heal itself, it sends wound-repair cells to the site. Those cells invade the matrix, take up the gene, and express the protein during the entire healing process. If a gene-encoding platelet-derived growth factor b is used, the expressed protein acts as a chemoattractant for other cells to come to the site, Sosnowski says.

The matrix can be used to accelerate the wound-healing process in different types of tissues by changing the gene. Selective Genetics is using the gene-activated matrix to stimulate diabetic ulcer wound healing, bone formation, and blood vessel formation, Sosnowski says.

"What's really nice about the technology," Sosnowski explains, "is that it remains localized at the desired site. Not only is the gene contained within the injured site, but if genes encoding proteins with cell retention signals are used, the protein also remains localized. The protein doesn't wander outside of that local environment."

Some of the work in nonviral gene delivery can be thought of as trying to create an artificial virus, without the viral components. DNA-lipid complexes already look morphologically like viruses, Huang points out.

"Most of the time in viral systems, we worry about taking things out, taking away, or trying to ameliorate any of the deficiencies the virus has, masking immunogenicity," Paul says. "We spend a lot of time trying to take away natural properties of the virus. In the nonviral systems, what we're essentially doing is creating synthetic viruses piece by piece, by adding in one element at a time to get the transfection more efficient, more targeted, able to escape any sort of nonspecific inflammation or immune response." Perhaps the vectors of the future will be hybrids, as researchers from the viral and nonviral sides meet in the middle.

Despite promising results in the clinic, DNA delivery without viruses still represents only a fraction of the research in gene therapy. But that may be changing.

"I already see people shifting from viral to maybe something in between viral and nonviral," Rolland says. "I would say that it will take some success in the clinic first to make people convinced that synthetic gene delivery systems are going to be the way to go. Some people are very strong supporters of nonviral systems and are encouraged by some recent tantalizing clinical results. However, until unequivocal success of pivotal trials in the clinic, people are going to be doubtful."

Gene Therapy: Safer and Virus-Free?

(Ferber, 2001)

New vectors for gene therapy aim to mimic viral vectors' pros without their dangerous cons.

If fields of science go through life stages, then childhood ended abruptly for gene therapy on 17 September 1999, when a teenage volunteer named Jesse Gelsinger died in a gene therapy clinical trial at the University of Pennsylvania in Philadelphia. Sunny talks describing future therapies for genetic diseases were replaced by public scrutiny, congressional hearings, and new rules. Gelsinger's death was blamed on an out-of-control immune response to the virus physicians had used to ferry the useful gene into tissue, and it prompted a hard look at the safety record of so-called viral vectors. It also spurred renewed interest in nonviral methods to deliver genes, methods that have been quietly gathering steam for more than a decade. Today, no gene therapy using any type of vector has been approved for clinical use. But researchers are working doggedly to develop methods that will deliver useful genes safely, to the right spot, and turn them on and off at will. Originally envisioned as treatments for hereditary diseases, gene therapies are now being developed to prevent and treat infectious diseases, cancer, heart disease, and other ailments. All of them rely on a gene's ability to produce a key protein when and where it's needed.

Viruses such as adenovirus and retrovirus are still the most popular vectors in lab studies and clinical trials. Viruses are well suited to gene delivery: They've evolved to home in on specific tissues, invade cells, and manipulate the cell's machinery to make viral proteins. But often they can be injected into a person only once or twice before the immune response they provoke poses a

safety threat, as in Gelsinger's case. That response can also destroy the viral vector or the cells it infects, blocking production of the useful protein. A spate of recent work has suggested that genes can be delivered effectively without using viruses. Most nonviral vectors fly under the radar of the immune system, and they're cheaper and easier to manufacture than viral vectors. But most of them have not been as efficient as viruses in shuttling genes into cells, and the genes that were delivered didn't remain active for long. That has begun to change in the past few years.

In the race to develop a reliable gene delivery method, researchers are putting money on a wide array of vectors, and so far no single method has taken the lead. Gene therapist Malcolm Brenner of Baylor College of Medicine in Houston, who is president of the American Society of Gene Therapy (ASGT), suspects that both viral and nonviral gene transfer methods will be needed, depending on the disease being treated. "Nobody has the perfect vector," he says. "What we're looking for is horses for courses."

DNA, Naked and Otherwise

Back in 1989, when human gene therapy was still a dream, dogma had it that viruses were the best and perhaps only way to ferry therapeutic genes into animal tissue. But Jon Wolff, a gene therapist at the University of Wisconsin, Madison, suspected otherwise. Philip Felgner, then at Vical, a San Diego biotechnology company, had just devised a way to shuttle genes into lab-grown animal cells by coating them with positively charged lipids-basically, shrink-wrapping the DNA. The charge helps the construct, called a lipoplex, stick to cell membranes and pop genes inside the cell. Wolff tested the method in animals, injecting mice with RNA lipoplexes and then checking their tissue for the presence of an enzyme encoded by the RNA. To his surprise, mice injected with lipid-coated RNA failed to activate the gene. But the control mice, which had been injected with uncoated, or "naked," RNA, did crank out the enzyme. "I thought my technician screwed up and reversed the two samples," Wolff recalls. "But he repeated it, and it kept getting better and better." Wolff was equally surprised a few months later, when genes ferried into muscle cells by loops of DNA called plasmids were expressed for weeks at a time. Researchers had thought that the only way to get long-lasting gene expression in animal tissue was to use a virus whose DNA stitched itself into the chromosomes of the recipient cells. But somehow, the naked plasmid DNA stuck around inside muscle cells, and the genes turned on and stayed on. "Even now I'm amazed," Wolff says. Naked DNA injections are still the simplest nonviral gene delivery method and so far one of the most successful.

Following Wolff and Felgner's early report, researchers quickly applied the naked DNA approach to a practical problem: building better vaccines. The method entails injecting a plasmid that encodes a protein from the unwanted microbe; the protein then provokes an immune response that would stop an infection. So far, clinical tests have been promising. For example, Stephen Hoffman, then of the Naval Medical Research Institute in Bethesda, Maryland, and his colleagues reported in 1998 that injections of plasmid DNA encoding a protein from a malaria parasite provoke a strong immune response in humans (Wang *et al*., 1998). And earlier this year, Harriet Robinson of Emory University in Atlanta and colleagues reported hat a naked DNA vaccine helps confer immunity in a monkey model of AIDS (Amara *et al*., 2001). Naked DNA therapies are also being tested against heart disease, cancer, and other disorders. The late cardiologist Jeffrey Isner of Tufts University School of Medicine in Boston and his colleagues developed a gene therapy for patients with coronary artery disease. The team injects a gene called VEGF, which boosts blood vessel growth, directly into patients' heart muscles by threading a special catheter through the arteries much as a surgeon would during angioplasty. The treatment gave promising results in a small phase I trial: New arteries sprouted from existing arteries, detouring blood around blockages to supply the heart muscle, according to work presented last week at the annual meeting of the American Heart Association. The technique, which is owned by a company Isner founded, called Vascular Genetics Inc. in Durham, North Carolina, apparently eases the severe pain of heart disease and improves pa-

tients' ability to exercise on a treadmill. The treatment could one day offer an alternative to bypass surgery, Isner told Science shortly before his death on 31 October, and a similar method could help save the legs of diabetes patients and others whose circulatory disease is so severe they are candidates for amputation.

But naked DNA injections haven't worked well to deliver genes to tissues other than liver and muscle. To sneak genes into other tissues, researchers have tried coating the DNA with different combinations of lipids and polymers, which have been shown by trial and error to help cultured cells take up DNA. Some such therapies are now being tested in the clinic. For example, Vical researchers have developed a lipid-coated plasmid that is injected directly into tumors to deliver the HLA-B7 gene; this gene encodes a protein that sparks an immune response against the tumor. In a phase II trial, the immune response shrank tumors and prolonged life in eight of 73 patients with aggressive melanoma who had failed to respond to other treatments, company collaborators reported in May at the annual meeting of the American Society of Clinical Oncology.

A different gene delivery strategy for head and neck cancer- composed of a gene called interleukin-2 coated with cholesterol and a synthetic lipid- also gave promising results in a phase II trial in patients with tumors that could not be surgically removed, a team from Valentis Inc. of Burlingame, California, reported at the ASGT annual meeting in June. The treatment kept cancer from spreading for more than 4 months when combined with traditional chemotherapy- 38% longer than patients receiving chemotherapy alone.

Up-and-Coming Vectors

The number of clinical trials using nonviral vectors for gene therapy is growing, but many diseases can't be treated using the nonviral gene delivery methods that are farthest along. That's because most methods have delivered only low levels of active genes for short periods of time. Researchers are currently hammering out other approaches in the lab. They're trying to improve upon current vectors by finding ways to penetrate a higher percentage of cells in target tissues and make imported genes last longer once inside the cells. Short-lived gene expression is fine for vaccines, cancer therapies, and angiogenesis. Indeed, Isner called it "a major-league safety advantage" for vascular gene therapy, because only temporary gene expression is needed to grow new vessels, and because insertion into the genome- the goal of some viral-based gene therapies-could disrupt other genes, possibly causing cancer. But to treat other diseases, therapeutic genes might have to pump out more protein for longer periods. Today's viral vectors still do this better than nonviral ones do, but lab experiments with new nonviral methods are closing the gap. For example, a method called electroporation, developed by immunologist Richard Heller's team at the University of South Florida in Tampa, transfers genes more than 80 times as efficiently as naked DNA injections. The team injects DNA into the target tissue-usually skin, muscle, or tumors-and uses a specially designed electrode to apply an electric field, which punches temporary holes in cell membranes that allow DNA into the cell. The method hasn't been tested in the clinic, but it's close: Gene therapist Lou Smith of Valentis and his colleagues recently used electroporation to transfer a blood-clotting gene to hemophiliac dogs, temporarily eliminating symptoms of the disease, according to work presented at a meeting in May sponsored by the National Hemophilia Foundation. And Heller's team reported at the June ASGT meeting that the method helped deliver a cancer-fighting gene called interleukin-12 into skin tumors, causing some of them to disappear in mice. They're now testing the method to see if it can provoke an immune response powerful enough to clear tumors in animals with melanoma.

Another novel nonviral strategy, developed by geneticist Richard Selden's team at Transkaryotic Therapies in Cambridge, Massachusetts, also improves gene-transfer efficiency. Instead of ferrying genes into cells inside the body, the researchers remove cells, insert genes, grow lots of modified cells in the laboratory, and then inject the cells into the abdominal cavity. The researchers used the method to transfer a gene encoding a blood-clotting protein called factor VIII into skin

cells taken from six hemophiliacs, they reported in the 7 June issue of The New England Journal of Medicine. When the cells were returned to the body, they produced the clotting protein. Four of the six patients needed less of their usual injected form of clotting protein and exhibited less bleeding for up to 10 months after the injection.

Long-lived gene expression has proved elusive for most nonviral vectors, in part be cause none of them stitch the useful gene into the genome of the host cell. But Mark Kay's team at Stanford has recently devised the first nonviral vector that has this power. Two plasmids are simultaneously injected into the tail vein of a mouse. One plasmid includes a therapeutic gene connected to pieces of a transposon, or jumping gene. The second plasmid encodes an enzyme that helps the hybrid gene on the first plasmid jump into the chromosome. When both plasmids were simultaneously injected, they sewed a key blood-clotting gene into liver cells of hemophiliac mice, where it pumped out enough protein to allow blood to clot normally, the team reported in the May 2000 issue of Nature Genetics. Kay's team also happened on a new way to achieve long-lived expression by delivering linear DNA fragments that don't insert themselves into the genome. These fragments persist in mouse liver cells for at least a year-about half the lifetime of a mouse, the team reported in the March issue of *Molecular Therapy*. "The persistence issue is being solved," Kay says. To get these long-lived plasmids into the liver, Kay used a method called hydrodynamics, developed by Wolff's team and Dexi Liu's team at the University of Pittsburgh. The method involves quickly injecting the tail vein of a mouse with naked DNA in a huge volume of saline, roughly the entire blood volume of the animal. The pressure somehow forces DNA out of blood vessels in the liver, where many of the liver cells take up and express the foreign genes.

No one proposes injecting people with a proportional amount- nearly 5 liters- of DNA-containing saline. But hydrostatic pressure could still help deliver genes to human tissue. For example, Wolff's team injected DNA into arteries that feed the arm and leg muscles of rhesus monkeys, using a blood pressure cuff to temporarily increase blood pressure. As they reported in March in *Human Gene Therapy*, the method delivers a reporter gene to about 30% of the muscle cells- a level of efficiency that rivals that of viral vectors. Wolff's team and colleagues at a company he founded, Mirus Corp. (a subsidiary of PanVera Corp. of Madison, Wisconsin), and at Transgene of Strasbourg, France, are planning a small clinical trial next year to see whether the pressure-cuff method can replace a defective muscle gene in young adults with Duchenne muscular dystrophy. Surgically clamping blood vessels does the gene-delivery trick, too, and can reach muscles that are inaccessible to a pressure cuff. In the July issue of *Molecular Therapy*, Leaf Huang's team at the University of Pittsburgh reported inserting a key gene to repair the diaphragm muscle of mice with muscular dystrophy (MD)- a crucial target because many MD patients die of suffocation when their diaphragm muscles fail to pull air into the lungs. The researchers surgically clamped the outgoing blood vessel for a few seconds, raising the blood pressure enough to deliver the therapeutic gene; Huang suspects that similar clamping methods could help push therapeutic genes into other organs as well. Whether or not it can be adapted to the clinic, hydrodynamics proves that high efficiency gene transfer is possible without viruses, Kay says. It's also the first method to rapidly pinpoint the best candidate genes for gene therapy. Researchers create small pools containing different genes, inject each pool into mice, and see quickly which contains a gene that helps treat the disease. With their candidates thus narrowed down, researchers can inject mice with each gene in the pool to identify which one helped. That's much quicker than cloning each candidate gene into a viral vector, and it could be important for diseases such as cancer, in which no one's sure which genes will prove effective. Liu says that the discovery of new therapeutic genes, together with more efficient delivery, "will make the field jump."

Vectors Tailored to Tissues

When viral gene therapy vectors are injected into the bloodstream, the viruses protect their gene payload, home in on their target tissue, and deliv-

er the genetic goods- as viruses have been doing for eons. Some researchers are devising complex nonviral vectors that act more like viruses, using tools developed by a generation of drug delivery specialists. The long-term goal is to transfer genes to the correct tissue to produce the desired clinical effect, says drug delivery specialist Sung Wan Kim of the University of Utah in Salt Lake City. Custom-designing vectors, Kim says, relies on several strategic decisions: whether to inject into the bloodstream or directly into the tissue; which combination of polymer, lipid, and other molecules to use for a particular tissue; and whether to attach another molecule to help target the complex to the correct cells. Despite the complexity, it's beginning to work: In the August issue of *Gene Therapy*, Kim's team reported a three-part system called TerplexDNA that delivers genes to rabbit heart tissue 20 to 100 times more efficiently than naked DNA. The vector includes DNA, a positively charged polymer to help protect DNA from enzymes that would chop it up, and a lipid that heart muscle cells recognize and take up. The team has also developed a way to deliver useful genes by injection into the bloodstream. The method uses DNA wrapped in a soluble, degradable polymer to target white blood cells. In the July issue of *Gene Therapy*, the team reported that one injection in mice helped deliver two genes to white blood cells throughout the body. They pumped out proteins that made their way to the pancreas and blocked the autoimmune reaction believed to cause juvenile diabetes.

Gene therapist Leonard Seymour's team at the University of Birmingham, U.K., has developed another way to ferry genes through the bloodstream to target tissue: cloaking the genes in a two part polymer shell and freeing them where they're needed. A polymer called polylysine packs the DNA into small particles, and a second polymer makes it slippery and able to evade immune proteins and cells. Once inside the target cell, the chemical environment causes the polylysine to break apart, liberating the DNA for expression. "It works amazingly well," Seymour says. Eventually, the team would like to add guidance molecules such as a specific antibody, peptide, or sugar-that are recognized and taken up only by particular tissues, making targeted delivery possible.

Complex nonviral carriers are a long way from the clinic, but they may offer a glimpse of future gene therapies. Years from now, gene therapy vectors might be a sort of semisynthetic virus, combining the best of today's viral and nonviral carriers, ASGT president Brenner predicts. Such a vector would make precise and permanent fixes to genetic defects that underlie disease by homing in on a specific tissue and replacing or fixing a defective gene, while safely avoiding the potential dangers of viral vectors. But other experts see a different future, in which genes are given temporarily and produce a precise dose of protein for just as long as it's needed. In short, says Felgner, "the idea would be to inject genes like any other drug."

Repair Kits for Faulty Genes

A balky appliance forces a choice: repair or replace. Defective genes impose the same choice. Most gene therapists have gone the replacement route, providing intact genes to make up for the defective version nature provided. But a few researchers are developing molecular toolkits to correct mutations in the genome. These so-called molecular targeting approaches don't touch the stretches of DNA flanking the faulty gene that help regulate its expression, so after the gene is repaired, the cell can stilt property control when and how much protein the gene produces. That differs from gene replacement approaches, which don't necessarily replace all the normal expression signals. This strategy could make the difference in treating diseases that require the right amount of therapeutic protein at the right time. So far, gene-repair methods have corrected mutations involving the insertion, deletion, or substitution of only a handful of nucteotides at a time, and only a few of the methods have been tested in animals. But the following techniques offer potential means to achieve a longtime dream of gene therapists: a lasting cure for genetic disease.

Triplex-Forming Oligonucleotides (TFOs)

These snippets of single stranded DNA recognize double strands of DNA with identical or nearly

identical sequences and nestle themselves into the double helix there to form a triple helix, or triplex. There are two versions of the method, one of which corrects mutations and the other of which purposely introduces mutations that stop production of a dangerous protein. To correct mutations, the TFO is linked to another snippet of DNA, this one double-stranded, that has the correct sequence of the defective gene. The double-stranded fragment shuffles itself into the genome near where the TFO has bound, replacing the misspelled portion of the gene. To stop production of a protein, a single-stranded TFO is used alone, without a linked fragment of DNA. It snuggles into the misspelled portion of the gene, forming a triplex. The cell's repair enzymes are attracted to the triplex but don't know how to fix it. Instead they make new mistakes, introducing random mutations into the target gene. One drawback: TFOs work only on the minority of genes that have DNA sequences capable of forming triple helices.

Small Fragment Homologous Replacement

This method takes advantage of the cell's ability to shuffle different copies of a gene by exchanging stretches of DNA between chromosomes, a process called homologous recombination. It uses a 400- to 800-base DNA fragment that's identical to part of the defective gene, except for the stretch that's to be repaired. The cell exchanges the fragment into one or both chromosomes. In the August issue of *Gene Therapy*, Dieter Gruenert's team at the University of Vermont in Burlington reported fixing a mutation that hampers breathing in mice with cystic fibrosis.

Viral Gene Targeting

Gene therapists usually use adeno-associated virus to deliver intact genes to replace defective copies. But apparently the virus can also be used to repair defective genes in the chromosome. Part of a normal gene is stitched into the single stranded viral DNA, and the cell's repair machinery uses it to correct the mistake in its own genome. So far, the technique has repaired a variety of mutations in cultured human cells, including nucleotide deletions, insertions, and substitutions.

Chimeraplasty

Sickle cell anemia and many other genetic diseases are caused by misspellings of a single nucteotide in a single gene. In this approach, researchers create dumbbell-shaped hybrid molecules, part DNA and part RNA, that contain the correct spelling of the gene; the molecules seem to bind to the misspelled portion of the genomic DNA and fix the mistake. But this technique has met with hard questions since it was introduced in the mid-1990s. "It's fair to say there's been some controversy with regard to reproducibility," says molecular biologist Peter Glazer of Yale University School of Medicine. A handful of researchers defend the method, but few are pursuing it.

Viral Vectors Still Pack Surprises

(Marshall, 2001)

Viruses may be lowly parasites, but their power to invade cells has won them a big part in gene therapy. Stripped of disease-causing elements, they work as natural syringes to inject DNA into human cells. Such "viral vectors" now dominate gene therapy: Nearly three quarters of all protocols use them. Even so, researchers view their parasitic past with suspicion and worry about unforeseen problems in the clinic. The tamest viruses have produced surprises, as researchers using adeno-associated virus (AAV) learned recently. In September, federal overseers asked Stanford University's Mark Kay to put "on hold" a clinical trial using an AAV vector to treat hemophilia B, an inherited blood disorder. The reason: Signs of AAV in the patient's semen raised a concern that gene therapy might have changed the man's inheritable DNA. It's not unusual to detect traces of a

vector after gene therapy, Kay says. But in this case, the signal persisted "at a low level" for weeks before it cleared, he says. Kay alerted the Recombinant DNA Advisory Committee (RAC), an oversight group at the National Institutes of Health (NIH), and the Food and Drug Administration (FDA). The FDA asked for a pause; the case will be discussed in the RAC on 5 December. The RAC forbids any gene therapy that changes the "germ line"—eggs or sperm—either inadvertently or for genetic enhancement, because germ line mutations could be passed on to future generations. Kay already takes steps to prevent inadvertent alterations. His team informs patients that there is a small risk of germ line changes and, before therapy, offers to bank the sperm of mate patients and asks them to use barrier contraception until their semen is clear of vector signal. Kay doubts that germ tine changes occurred in this hemophilia patient. Instead, he thinks the AAV signal probably came from typical "shedding" of vector seen in body fluids. But he hopes the RAC discussion will lead to a consensus on risk. "We're changing germ lines at the time in cancer therapy" with DNA-mutating chemotherapy- and that doesn't bother people, Kay notes. But he understands that gene therapy is "new territory." He favors guidelines that would allow these safety trials to continue if the probability of germ line alteration remains low.

Widely regarded as ultrasafe, AAV ran into another hurdle earlier this year. Although wild-type AAV infects many people, it doesn't seem to cause illness. But researchers got a scare last winter when mice that had been injected with an AAV vector developed liver tumors. This discovery prompted a short pause in two clinical trials using an AAV vector and an inquiry by U.S. health agencies in March. A joint review by FDA and RAC concluded that the AAV vector probably did not cause the mouse cancers. Clinical trials using AAV have resumed. The cancer scare arose when molecular biologist Mark Sands of Washington University in St. Louis, Missouri, was reviewing data on mice in a gene therapy test. Sands is developing an AAV vector to treat people with inherited enzyme deficiencies, concentrating on a fatal disorder called mucopolysaccharidosis type VII, in which the body fails to process waste in lysosomes. Sands created knockout mice with this disorder and successfully treated them with AAV-vector gene therapy. But during a routine pathology review last year, he discovered that three of five mice sacrificed late in life-at 18 months, the human equivalent of 55-year-oldshad massive liver or blood vessel tumors. "It scared me. I had never seen tumors like this," says Sands, although he had used identical mice in many experiments-and this particular group of 59 had seemed tumor-free until the end of the study. On reexamination, three additional animals, the youngest sacrificed at 8 months, were found to have had tumors. Sands was concerned that the AAV vector might have inserted new genes into the mouse DNA in a way that triggered cancerous growth. After reviewing the data, experts at a joint FDARAC meeting in March ruled out "insertional mutagenesis" as a cause of cancer. Sands agrees. But that does not rule out other possible vector-induced changes, Sands notes.

What actually caused the cancers remains unclear. Some panel members suggested that the knockout mice may have been prone to liver cancer. R. Jude Samulski of the University of North Carolina, Chapel Hill, a vector expert who took part in the RAC review, suggests that when these mice are cured of their inherited enzyme disorder, another genetic flaw may cause cancer in old age. But Sands hasn't seen evidence that the mice are prone to cancer. And it troubles him that other researchers have not allowed mice to live as long as he did for safety testing. Although the scientific puzzle remains unsolved, Mark Kay and Terence Flotte, a gene therapist at the University of Florida, Gainesville, are confident that AAV vector can be used safety in gene therapy. The NIH and FDA, meanwhile, have asked Sands to do another mouse study to see if he can repeat the results. The research will require "hundreds" of animals, he says, and "years" to complete.

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