Nonviral Strategies for Gene Therapy

Many drawbacks of viral gene delivery agents might be overcome by nonviral systems. Studies in patients suggest these systems have potential as therapies and as vaccines

by Philip L. Felgner

As Theodore Friedmann notes in “Overcoming the Obstacles to Gene Therapy” on page 96 of this issue, many efforts at developing gene therapy employ modified viruses to shuttle into human cells genes coding for potentially therapeutic proteins. The aim is to induce cells that are invaded by a virus to transfer the gene to the cell nucleus. The cells should then “express,” or manufacture, the needed protein specified by the gene.

Viruses are effective at transferring genes into cells because they have evolved specialized mechanisms that allow them to bind to specific types of cells and to deliver their cargo efficiently into the cellular interior. Yet the therapeutic use of viruses as gene delivery vehicles, or vectors, entails problems. Some viruses can disrupt the DNA of the cells they infect, with potentially harmful results. Furthermore, weakened viruses can conceivably change inside the body and regain their pathogenic activity. An additional serious limitation is that a patient may generate an immune response to the microbe. Such responses can quickly make a gene therapy useless, because they may either destroy the virus itself or possibly kill the infected cells before the therapeutic gene has a chance to help a patient.

For these reasons, researchers have long wanted to deliver therapeutic genes to cells without using infectious agents. Further, physicians have come to realize over the past five years or so that for many of the conditions that might ultimately be treated by gene therapy, repeated treatments will probably be needed, rather than a one-time procedure aimed at producing a permanent cure. Nonviral techniques could be especially suitable for repeated use, because they do not elicit the immune responses that can damage viral vectors.

One approach I have studied, which is now being tested in humans, employs complexes consisting of DNA and nonimmunogenic lipids. Recent years have also seen the surprising discovery that injecting “naked” DNA into experimental animals and patients can provoke expression of encoded proteins. This second approach, as will be seen, holds particular promise for new vaccines.

Electrical Problems

Scientists have long been enchanted by the possibility that they could alter cells in selected ways by putting foreign DNA into them. John Holland of the University of California at San Diego and several of his contemporaries showed as long ago as the mid-1950s that cells could take up nucleic acids (RNA or DNA) extracted from viruses and express them as proteins. This discovery provided an incentive for scientists to improve the efficiency of gene “transfection,” the delivery of functional genes to cells.

During the 1960s, investigators established that a principal obstacle to the uptake of DNA by cells was that in water solution, such as the milieu that bathes cells in the body, the molecule has a negative electrical charge.
Gene Therapy

This means it tends to be repelled from the membranes of cells, because they are also negatively charged. Researchers therefore developed techniques that combined DNA with chemicals that electrically neutralized it and so allowed it to be absorbed more easily. One technique made use of a positively charged organic polymer called DEAE-dextran. Another procedure employed the mineral calcium phosphate.

Researchers established by these means that human cell cultures could take up genes and permanently express them. One important demonstration employed the gene for the enzyme thymidine kinase, isolated from the herpes simplex virus. Some cells can incorporate the gene when it is complexed with calcium phosphate and produce the enzyme in a stable fashion.

In the late 1970s the modern biotechnology industry got its start with the discovery of techniques for taking individual genes from cells and splicing them into plasmids—flexible loops of DNA that multiply naturally within bacteria. Recombinant DNA techniques therefore allowed investigators to produce large numbers of copies of specific genes. Paul Berg and his colleagues at Stanford University melded recombinant DNA and chemical cell transfection technology by delivering recombinant plasmids derived from bacteria into cultured mammalian cells. Expression of genes spliced into such plasmids suggested the genes were being taken to the nucleus, because genes usually cannot be expressed unless they are acted on by nuclear proteins. These nonviral transfection methods were then applied to produce many of the mammalian cell lines that industry uses today to produce medicinal recombinant proteins, such as the Factor VIII administered to hemophiliacs to correct potentially life-threatening blood-clotting problems.

Lipids to the Fore

Despite the commercial value of chemical techniques, most investigators considered them too inefficient for gene therapy. Yet Berg and Demetrios Papahadjopoulos of the University of California at San Francisco also successfully transfected cells with genes by exposing the cells to gene-containing liposomes, which are minute hollow spheres composed of a lipid (fatty) membrane on the outside and an aqueous solution within. Claude Nicoloau of Harvard Medical School achieved the same result. This work also suggested that the genes of the plasmids were getting to the nucleus, where the cell’s machinery was acting on the introduced genes and causing them to be expressed as proteins.

First described in the 1960s by Alec D. Bangham of the Babraham Institute in Cambridge, England, liposomes can form spontaneously....
when certain types of lipids are suspended in an aqueous solution. Liposomes resemble animal cells in that the outer membrane consists of a double layer of lipid molecules. This feature arises because the lipid molecules that are employed have a water-loving and a water-hating end. In water solutions they form double-layered membranes in which the water-loving heads face out toward the aqueous external environment and also in toward the water-filled center of the liposome. This cell-like aspect had long suggested to investigators that liposomes "loaded" with some medicinal substance might fuse with cells and deliver the liposomes' contents into the cellular interior.

The results with liposomes were encouraging, but a technical problem slowed their practical exploitation for delivering plasmids into cells. The internal diameter of a liposome—between about 0.025 and 0.1 micron—is typically much less than the longest dimension of a DNA plasmid, which may be as much as two microns. The mismatch meant that when liposomes were synthesized in the presence of plasmids, only a few tightly wound plasmids were encapsulated.

Nevertheless, optimistic scientists believed ways could be found to improve the encapsulation rate. Working at Syntex Research in Palo Alto, Calif., my colleagues and I theorized that we might be able to modify liposomes to capture plasmids more effectively and to deliver more readily their contents into cells.

Our idea was to make liposomes in which some of the standard lipids were replaced by ones carrying a positive charge at the water-loving end. This, we thought, should make the liposomes interact more easily with DNA and RNA, as well as with cell surfaces. At the time, however—1983—there were few examples of positively charged (cationic) lipids that had the right shape to organize into liposomes.

We therefore synthesized variant cationic lipids that we predicted would have the right properties. The molecules behaved as expected, and the resulting cationic liposomes bound firmly to the surface of cells in tissue culture. Furthermore, simply by mixing plasmids with about eight times their mass of cationic lipids, we could effectively capture all the DNA. I was pleased to find that I could readily create conditions that produced physically stable complexes.

Exploiting Discovery

The structures formed from mixtures of plasmids and cationic lipids are more variable and complicated than simple liposomes. We often find plasmids enclosed in tubelike lipid structures, for example. And under the right conditions, a lipid tube containing a plasmid can fold up to form a dense particle with a lipid wall. This structure is somewhat like that of some viruses. Because structures formed from cationic lipids are so different from simple liposomes, researchers have recently agreed to give them a new name: lipoplexes.

I had expected to have to modify lipoplexes further before they would
LIPIDS in lipoplexes help DNA enter a cell (left and front), before cellular organelles take up the lipids. Naked-DNA plasmids (right) can occasionally enter a cell through a tear.

deliver their payload into cells. But, remarkably, an undergraduate intern in my lab showed that these rudimentary structures transfected cells at a significant rate. Mixing cationic lipids with DNA has since become a standard technique for inserting genes into cultured cells, and many cationic lipid preparations are now available commercially for this purpose.

Researchers have lately initiated studies of lipoplexes in humans. The first candidate therapy consists of lipoplexes containing genes for an immune system protein known as HLA-B7, a so-called major histocompatibility antigen. When cancer cells express HLA-B7, they stimulate a patient’s immune system to recognize them as foreign and selectively destroy them. In clinical protocols sponsored by Vical, more than 90 patients who had failed to respond to standard cancer treatments were given injections into their tumors of lipoplexes containing DNA encoding HLA-B7.

In most cases, investigators could demonstrate that the treatment stimulated production of HLA-B7 protein. Sixty of the patients suffered from malignant melanoma. In about a third of these cases the lipoplex-injected tumor either shrank or disappeared. Advanced melanoma often spreads widely throughout the body, so injecting drugs into visible tumors may not cure most cases. But our encouraging preliminary findings indicate that lipoplexes might help treat melanoma. Sometimes treatment with lipoplexes makes even uninjected tumors shrink. Other studies with lipoplexes containing the gene for HLA-B7 aim to establish the safety and efficacy of similar treatments for inoperable colon, kidney and breast cancer.

Vical is also sponsoring a clinical trial of a lipoplex formulation containing the gene for the immune system protein interleukin-2 (IL-2). IL-2 is infused into patients to treat kidney cancer, but it has serious toxic side effects. Injected into a tumor, lipoplexes might stimulate the immune system by producing locally high concentrations of IL-2 while avoiding most of the toxic side effects.

Additionally, Genzyme General Division is studying lipoplexes in patients with cystic fibrosis. For treating that disease, the structures carry a gene for the protein that is defective in the illness—the cystic fibrosis transmembrane conductance regulator. The lipoplexes are delivered with an aerosol spray into a patient’s lungs, where expression of the protein should mitigate some of the most serious symptoms.

The levels of gene expression obtainable in animals with lipoplex formulations are in some instances comparable to the levels obtainable with viral gene delivery systems. Still, the efficiencies of the two systems differ substantially. Some viruses are almost 100 percent efficient at delivering their genome into cells, so that 1,000 viruses can infect almost 1,000 cells of the right type. To transfect 1,000 cells with lipoplexes would require about 10 million copies of the gene in a comparable number of lipoplexes, making this approach some 10,000 times less efficient.

One strategy we and others are pursuing to improve the efficiency of lipoplexes is to incorporate into their outer surface specialized proteins or protein fragments resembling those that target viruses to particular cell types. We might also include other molecules to facilitate gene survival and functioning in transfected cells. For example, so-called membrane fusion proteins of viruses, which normally help viruses escape from the cell’s internal waste disposal system, could be incorporated. In addition, researchers are experimenting with ways of attaching to genes certain viral proteins, known as nuclear targeting signals, that help to direct viral genes to the cell nucleus.

Meanwhile we and others are also pursuing applications for naked DNA. In the late 1980s, long before the lipoplexes had entered clinical trials, my colleagues Jon A. Wolff of the University of Wisconsin and Robert W. Malone and I, along with some others, made a surprising discovery. Hoping to determine which lipid formulations were most effective at gene delivery to cells, we had decided to measure the expression of a particular, easily monitored gene after we injected various mouse tissues with lipoplexes carrying that gene.

It was a memorable day when we analyzed the data from our first experiment and saw we had obtained levels of gene expression in skeletal muscle comparable to the best results we had obtained by transfecting cells in culture. But the next observation was unprecedented. We found that DNA by itself, which we had employed in some tests as a control experiment, gave expression levels that were similar to or greater than the lipid formulations.

Naked DNA

W e repeated these experiments several times in our different laboratories, but the results always came out the same way: naked DNA, injected into the muscle of an animal, was expressed as protein. Moreover, we could achieve quite a high local concentration of the protein, up to 100 nanograms of the gene product per gram of muscle tissue.

Despite the electrical repulsion between cell surfaces and DNA, it appeared that a few cells were nonetheless able to assimilate the molecule. To this day, it is unclear how. Perhaps a small amount of tissue damage or increased pressure at the injection site plays a role.

In principle, it seemed that it might be possible to inject patients’ muscles with naked DNA that would then produce therapeutic amounts of a selected protein. There is a great need, for example, for better ways to deliver to patients insulin for treating diabetes, or the blood-clotting protein Factor VIII or IX for treatment of hemophilia. Yet in these early studies the locally high concentrations of protein produced inside the muscle would not have been sufficient to be effective against these diseases when the proteins were diluted into the three liters of plasma in the bloodstream.

Thanks to improvements in plasmid design, my colleagues and I have recent-
ly made progress. We find we can stimulate the production of red blood cells in mice by injecting the animals with naked plasmids encoding erythropoietin. This hormone is used to help patients grow new red blood cells after chemotherapy and radiation therapy. Perhaps in the future, injections of similar recombinant plasmids into muscle will be a less expensive alternative to delivering erythropoietin itself.

In the nearer term, naked DNA has promise for use in vaccines, because even minute amounts of protein can stimulate a protective immune response. Immunologic studies have indicated that proteins can elicit two different kinds of immunity. The first, humoral immunity, develops after a pathogen is destroyed by the immune system. The damaged microbes are then taken up by specialized cells that display the foreign proteins to antibody-secreting cells called B lymphocytes. The B lymphocytes respond by producing antibodies that recognize the specific foreign proteins. These antibodies will rapidly bind to the original pathogen whenever it is encountered again, neutralizing it or marking it for destruction by other components of the immune system.

The second kind of response, known as cellular immunity, occurs when invading pathogens colonize cells and force them to make more of the pathogen. Short pieces of the pathogen’s proteins are then displayed on the surface of the infected cell. The immune system responds by producing other lymphocytes: activated T lymphocytes that recognize these fragments. These lymphocytes do not produce antibodies but rather destroy directly cells that are displaying the critical fragments. If the pathogen invades the body again, infected cells display the fragments and so are destroyed.

Dennis A. Carson of U.C.S.D. and I reasoned that naked plasmid DNA, because it invades cells, might be able to stimulate cellular as well as humoral immunity. Our very first experiment, conducted with Gary H. Rhodes, showed that injecting into mice a plasmid encoding a coat protein from the human immunodeficiency virus (HIV) stimulated the mice to generate antibodies that bound to the HIV protein. Rhodes subsequently demonstrated that the plasmids elicited cellular immune responses, too. In laboratory tests, T lymphocytes from these animals attacked cells displaying fragments of HIV proteins.

Looking Ahead

The crowning achievement came when Sueanne E. Parker from my laboratory showed that plasmids carrying a gene from influenza virus could be used to immunize mice and so protect them from what would otherwise be a lethal viral dose. Margaret Liu and her colleagues at Merck have confirmed these findings in animals and expanded them into the development of a variety of potential DNA vaccines that produce long-lasting cellular and humoral responses. Merck has a naked-DNA influenza vaccine candidate in human clinical trials.

We can expect to see clinical testing of DNA vaccines against herpes, malaria and HIV in the not too distant future. In the longer term, tuberculosis, papilloma, chlamydia and hepatitis could be targets. As well as preventing infection, such vaccines might stimulate the immune systems of those already sick. A clinical trial of a naked-DNA therapy for lymphoma, a cancer of the white blood cells, is being planned.

Lipoplexes and naked DNA are not the only nonviral approaches to gene therapy. Investigators are also studying various nonlipid cationic polymers that form complexes with DNA. These structures, known as polyplexes, are showing promise in the laboratory and in clinical trials.

One important goal for nonviral gene therapy will be to develop delivery systems that can be injected into the bloodstream and will deliver their DNA sequences to the appropriate tissues, such as lung, liver, spleen or bone marrow. Gene delivery systems that can be swallowed as a pill could make gene therapy even more convenient. And if delivery systems could be made to target tumor cells specifically, they could in various ways improve the treatment of cancer. Ultimately, gene therapy could be employed to correct mutated genes in the cells of people with genetic diseases or cancer. A technique known as gene targeting offers a possible approach; it has been used successfully with lipoplexes in cultured cells.

Nonviral gene delivery to cells via lipoplexes, polyplexes and naked DNA is now an important and expanding field of pharmaceutical research and development. If the current pace of progress is maintained, coming decades should see many products based on this technology being administered on a routine basis for the treatment and prevention of common diseases.

The Author

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LIPOPLEXES to fight cancer are administered to a melanoma patient by Alfred E. Chang and Gary J. Nabel of the University of Michigan Medical Center.